Stimulation of Fibroblast Growth Factor Receptor-1 Occupancy and Signaling by Cell Surface–associated Syndecans and Glypican

Robert Steinfeld, Herman Van Den Berghe, and Guido David

Center for Human Genetics, University of Leuven and Flanders Institute for Biotechnology, B-3000 Leuven, Belgium

Abstract. The formation of distinctive basic FGF–heparan sulfate complexes is essential for the binding of bFGF to its cognate receptor. In previous experiments, cell-surface heparan sulfate proteoglycans extracted from human lung fibroblasts could not be shown to promote high affinity binding of bFGF when added to heparan sulfate–deficient cells that express FGF receptor-1 (FGFR1) (Aviezer, D., D. Hecht, M. Safran, M. Eisinger, G. David, and A. Yayon. 1994. *Cell.* 79:1005–1013). In alternative tests to establish whether cell-surface proteoglycans can support the formation of the required complexes, K562 cells were first transfected with the IIIc splice variant of FGFR1 and then transfected with constructs coding for either syndecan-1, syndecan-2, syndecan-4 or glypican, or with an antisense syndecan-4 construct. Cells cotransfected with receptor and proteoglycan showed a two- to threefold increase in neutral salt-resistant specific ^{125}I-bFGF binding in comparison to cells transfected with only receptor or cells cotransfected with receptor and antisyndecan-4. Exogenous heparin enhanced the specific binding and affinity cross-linking of ^{125}I-bFGF to FGFR1 in receptor transfectants that were not cotransfected with proteoglycan, but had no effect on this binding and decreased the yield of bFGFR cross-links in cells that were cotransfected with proteoglycan. Receptor-transfectant cells showed a decrease in glycophorin A expression when exposed to bFGF. This suppression was dose-dependent and obtained at significantly lower concentrations of bFGF in proteoglycan-cotransfected cells. Finally, complementary cell-free binding assays indicated that the affinity of ^{125}I-bFGF for an immobilized FGFR1 ectodomain was increased threefold when the syndecan-4 ectodomain was coimmobilized with receptor. Equimolar amounts of soluble syndecan-4 ectodomain, in contrast, had no effect on this binding. We conclude that, at least in K562 cells, syndecans and glypican can support bFGF–FGFR1 interactions and signaling, and that cell-surface association may augment their effectiveness.

THE signaling pathways that are activated by the binding of various FGFs, Vascular Endothelial Growth Factor (VEGF) and Heparin-Binding EGF-like growth factor to their cognate receptors have been qualified as "heparin dependent." This contention is based on the failure of these signaling systems in cells that are defective in the synthesis of heparan sulfate (HS)[1] and on the ability to restore the activity of these pathways in these cells by providing an exogenous source of heparin-like polysaccharide. In the case of basic FGF (bFGF or FGF-2), heparin restores the high affinity binding of the growth factor to the tyrosine kinase receptor proteins, and restores the biological effects of this growth factor on cell differentiation and proliferation (Yayon et al., 1991; Rapraeger et al., 1991). The primary defect in the HS-deficient cells appears to be situated at the level of the initiating event, with the growth factor failing to occupy a binding site on the receptor and to induce a receptor configuration that leads to signaling. Different models that have been proposed as explanations for this HS requirement and the pharmacological effects of heparin (reviewed by Mason, 1994) include: a heparin-induced fit, whereby the glycosaminoglycan allows the growth factor to adopt a conformation that is appropriate for receptor engagement (Yayon et al., 1991), the need for HS to participate in the formation of a multimolecular signaling complex, whereby it binds simultaneously to both ligand and receptor (Nugent and Edelman, 1992; Kan et al., 1993; Guimond et al., 1993; Pantoliano et al., 1994), and indirect effects of heparin on the receptor dimerization that is required for signaling, by promoting the formation of ligand dimers (Ornitz et al., 1992; Spivak-Kroizman et al., 1994). On the other hand, these concepts have also been challenged or

Please address all correspondence to Guido David, Center for Human Genetics, Campus Gasthuisberg, O&N6, Herestraat 49, B-3000 Leuven, Belgium. Tel.: 32 16 345863; Fax: 32 16 345997. e-mail Guido.David@med.kuleuven.ac.be

1. *Abbreviations used in this paper*: bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; GpA, glycophorin A; GPI, glycosyl phosphatidylinositol; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan.

amended, whereby heparin was shown to only moderately increase the affinity of the growth factor for its receptor (two- to threefold increase) and heparin or HS were proposed only to be needed at low concentrations of ligand (Roghani et al., 1994). In all models, the direct binding interactions between the growth factor and heparin-like glycosaminoglycan are proposed as essential for the activation of the signaling pathway.

bFGF binds preferentially to exon IIIc-containing forms of the FGF receptors (FGFRs) 1–3, which are predominantly mesenchymally expressed (Dionne et al., 1990; Johnson et al., 1991; Keegan et al., 1991; Miki et al., 1992; Yayon et al., 1992; Werner et al., 1992; Chellaiah et al., 1994). In vitro the affinity of bFGF for the IIIc splice variant of FGFR1 is increased by about one order of magnitude when heparin is added (Pantoliano et al., 1994), and in HS-expressing cells, the affinity of bFGF for the receptors (10^{-10}–10^{-11} M) is about two orders of magnitude higher than the affinity of the growth factor for cell-surface HS (10^{-8}– 10^{-9} M) (Moscatelli, 1987; Wennström et al., 1991). Clusters of IdoA(2-OSO_3)α1,4GlcNSO_3 units have been identified as bFGF-binding sequences in HS chains derived from human skin fibroblasts (Turnbull et al., 1992) and bovine aortic muscle cells (Habuchi et al., 1992). Heparin-derived penta- or hexasaccharides of similar structure effectively bind to bFGF and inhibit bFGF binding to cell surface HS proteoglycans (HSPGs), but fail to promote FGFR binding (Tyrrell et al., 1993; Maccarana et al., 1993). The minimal structural requirements to enhance bFGF binding to its receptor and to support bFGF-induced mitogenesis appear to be realized in a dodecasaccharide containing the bFGF-binding site and additional 6-O sulfated groups (Ishihara et al., 1993; Guimond et al., 1993; Walker et al., 1994).

The ability of cells to generate HS of a defined sequence complexity varies during ontogenesis (David et al., 1992*a*; Kato et al., 1994), and some observations directly imply that part of the cellular controls on signaling by FGF-like growth factors may occur at the level of the expression of the required HS cofactor/receptor sequences (Nurcombe et al., 1993). The possibility of PG specificity in this respect is supported by the observation that, in vitro, some heparins and whole PG extracts from human lung fibroblasts were able to induce the binding of bFGF to an immobilized recombinant FGFR–alkaline phosphatase fusion protein, whereas some specific PG forms, such as syndecans 1 and 2, that were purified from these cell extracts were inactive and even inhibited the effect of the active heparin fraction (Aviezer et al., 1994*a*). In subsequent studies, the major activating component of these PG extracts was identified as perlecan, the large extracellular matrix (basement membrane) PG (Aviezer et al., 1994*b*). These observations, together with indications that overexpression of syndecan-1 inhibits the bFGF-induced growth promotion of 3T3 cells (Mali et al., 1993), have lead to the suggestion that the local expression of active perlecan and the synergies and balances between activating and nonactivating classes of PGs will determine the degree and extent of bFGF-induced cellular responses (Aviezer et al., 1994*b*).

Our investigations were aimed at identifying cell surface–associated HSPGs that might promote bFGF binding and receptor activation. Using a cell system in which we were able to express independently the IIIc variant of FGFR1 and four types of PG (i.e., syndecans 1, 2, 4, and glypican), as well as a cell-free system in which we used 6xHis-tailed recombinant forms of receptor and PG to mimic their colocalization at the cell surface, we demonstrate that all these cell-surface HSPGs can support the bFGF–receptor interaction.

Materials and Methods

Plasmid Isolation and Construction

Clones for bFGF and FGFR1 were isolated from a human embryonic lung fibroblast λZAPII phage library using oligolabeled PCR-derived probes for bFGF and FGFR1 and standard screening procedures (Sambrook et al., 1989).

The bFGF clones were used as PCR templates. The primer set 5'-GGTGTCGACATCGAAGGTAGACCCGCCTTGCCCGAGGATGGC-3' and 5'-GGCCTGCAGTCAGCTCTTAGCAGACAT-3' used for the amplification reaction (Saiki et al., 1988) was designed to introduce unique restriction sites flanking the coding sequence and a Factor Xa cleavage site at the amino-terminal end. The PCR products were sequenced using an automated fluorescent sequencer (Pharmacia Biotechnology Benelux, Roosendaal, The Netherlands) and cloned into the prokaryotic expression vector pQE-9 (Qiagen, Chatsworth, CA), which introduces a 6xHis tag at the amino-terminal end of the encoded protein.

One FGFR1 clone, identified as the two Ig-like domain isoform (Ig II/IIIc) (Eisemann et al., 1991), was restricted with PstI to remove 370 bp of the 5' untranslated sequence and cloned into the eukaryotic expression vector pcDNA/Neo (Invitrogen, Leek, The Netherlands), using the HindIII and NotI sites from the multiple cloning sites of the vectors.

The cDNAs for human syndecan-4 (David et al., 1992*b*), syndecan-2 (Marynen et al., 1989), and syndecan-1 (Mali et al., 1990) were cloned into the KpnI and NheI sites of the episomal expression vector pREP4 (Invitrogen). The cDNA for glypican (David et al., 1990) was released with HindIII and NotI, and cloned into the corresponding sites of pREP4. A 630-bp fragment containing the complete coding sequence of syndecan-4 was antisense cloned into the HindIII and BamHI sites of the same vector.

Plasmids coding for 6xHis-tagged ectodomains of FGFR1 and syndecan-4 were constructed by PCR. A 300-bp fragment of FGFR1 was amplified using the primer set 5'-GACCCGCAGCCGCACATCCAGTGG-3' and 5'-CCGCTCGAGTCAGTGATGGTGATGGTGATGCTCCAGGTACAGGGGCGAGGTCATCACTGCC-3', digested with BfrI and XhoI, and cloned into the corresponding restriction sites of the FGFR1 plasmid, replacing the sequences that code for the transmembrane and cytoplasmatic domains. The resulting insert, FGFR1e, was cloned into pMEP4 via HindIII and XhoI. A sequence coding for a 6xHis-tagged ectodomain of syndecan-4 was constructed using the primers 5'-GCAATTAACCCTCACTAAAGGG-3' and 5'-CGCGTCGACTCAGTGATGGTGATGGTGATGCTCCGTTCTCTCAAAGATGTTGCTGCCCTGC-3'. The PCR fragment was restricted with BglII and SalI, and cloned in the corresponding sites of the syndecan-4 plasmid. The resulting insert, syn4e, was released with SpeI and XhoI, and cloned into the NheI and XhoI sites of the vector pMEP4. All constructs were sequenced to exclude mismatches.

Purification and Characterization of Recombinant bFGF

Nondenaturing purification of the recombinant 6xHis-bFGF was carried out according to standard protocols (Seno et al., 1990). In short, *Escherichia coli* M15 containing the appropriate pQE-9 construct plus the repressor plasmid pREP4 were induced with 1 mM IPTG at an OD of 0.9. Before sonication for 3 min on ice in the presence of 10 mM β-mercaptoethanol, the harvested cells were incubated for 1 h at 4°C in 50 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 15% sucrose, 0.1 mg/ml lysozyme, and 1 mM PMSF. After centrifugation, the bacterial lysate was applied to an Ni-NTA resin column (Qiagen), equilibrated at pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl), and eluted at pH 4.5 (50 mM NaH_2PO_4, 500 mM NaCl) (Hochuli et al., 1987). After readjustment to pH 8.0, this eluate was applied to Heparin-Ultrogel (IBF Pharmindustrie, Villeneuve-la-Garenne, France), washed with 1 M NaCl, and eluted with 2 M NaCl. Total yield was ~4 mg purified bFGF per liter of culture (20 g of cells), as determined

by colorimetric assay. The purified protein migrated as a [redacted]kD peptide in Tricine–SDS-PAGE (Schägger and von Jagow, 1987), and was detectable with an anti–bovine bFGF mAb on Western blot. Stimulation of thymidine incorporation in serum-starved Swiss 3T3 fibroblasts, induced with 2 ng/ml 6xHis-bFGF for 18 h, confirmed the biological activity of this recombinant product.

Extraction and Purification of Cell-surface PGs

Cell surface PGs were extracted with a Triton X-100 buffer in the presence of proteinase inhibitors, concentrated on a DEAE–Trisacryl M column (IBF Pharmindustry, Villeneuve-la-Garenne, France), and further purified by ion exchange chromatography on MonoQ in Triton-urea-Tris buffer (Lories et al., 1987). Immunopurification was carried out with core protein–specific mAbs immobilized on CNBr-activated Sepharose 4B (Lories et al., 1989).

Purification of Recombinant FGFR1 and Syndecan-4 Ectodomains

Recombinant 6xHis-tagged ectodomains of FGFR1 (FGFR1e) and syndecan-4 (Syn4e) were isolated from the conditioned culture media of K562 cells that were transfected with the corresponding episomal plasmid constructs. Serum-free media from pMEP4-transfected cells were harvested 12–16 h after induction with 5 μM $CdCl_2$. FGFR1e was purified by two consecutive absorptions on Ni-NTA resin (see above). Syn4e was first absorbed on DEAE–Trisacryl M and then purified by metal chelate chromatography. The final eluates were concentrated by ultrafiltration (Centricon 30; Amicon, Inc., Beverly, MA), and the quantity (~400 μg/liter medium) and purity (>90%) of the product were estimated by SDS-PAGE.

Western Blotting

Heparitinase and chondroitinase ABC–digested PG were fractionated by SDS-PAGE and blotted on Z probe membranes. The blots were first incubated with the designated mAbs, and then with alkaline phosphatase–conjugated second antibodies, and finally developed with AMPPD (Tropix, Bedford, MA) for chemiluminescence and autoradiography.

Analysis of the GAG Compositions

Free glycosaminoglycan side chains were obtained by proteinase K digestion of purified $^{35}SO_4$-labeled PGs. The GAG chains were either subjected to the low pH nitrous acid procedure (Shively and Conrad, 1976) or digested with chondroitinase ABC. Both preparations and an untreated control were precipitated with cetyl pyridinium chloride and then collected on glass filter papers. The HS content was calculated as $(cpm_{untreated} - cpm_{HNO_2})/cpm_{untreated}$, the chrondroitin sulfate (CS) content was calculated as $(cpm_{untreated} - cpm_{ABCase})/cpm_{untreated}$. All analyses were performed in duplicates.

Affinity Chromatography of HSPG on Chelate Complex-bound bFGF

Purified 6xHis-bFGF was reapplied to an Ni-NTA column at a concentration of 50–100 μg/ml gel (~1/100 of the maximal binding capacity), washed with assay buffer (50 mM Na_2HPO_4, pH 7.5, 0.1% Triton X-100, 20 μg/ml BSA) and increasing NaCl concentrations (0–2 M), and reequilibrated with assay buffer. No bFGF leakage could be detected during the wash. Immunopurified HSPGs were dialyzed against the assay buffer and applied to aliquots of bFGF–Ni-NTA resin. Bound HSPGs were eluted with a NaCl step gradient (0–2 M). Every chromatographic experiment was repeated at least once, with similar results.

Cell Transfections

K562 cells (ATCC CCL 243) were routinely grown in DME F12 medium supplemented with 10% FCS and L-glutamine. For transfection, K562 cells were prewashed with Ca^{++}- and Mg^{++}-free PBS and incubated for 10 min at 4°C (10^7 cells/ml Ca/Mg-free PBS) with 30 μg linearized FGFR1-pcDNA/Neo, or pcDNA/Neo, before electroporation at 240 V and 960 μF with a gene pulser (Bio Rad Laboratories, Richmond, CA). Selection was started 48 h later with 500 μg/ml G418. Stable transfection was achieved after 12 d, and subclones were established by two consecutive limited dilution procedures. Individual clones [redacted] characterized for specific ^{125}I-bFGF binding. The transfections with [redacted] episomal replicons pREP4[−], pREP4[Syn1], pREP4[Syn2], pREP4[Glyp], pREP4[Syn4], and pREP4 [antiSyn4] were performed in similar ways. Selection with 200 μg/ml of hygromycin over 2 wk resulted in stable cell populations that were not further subcloned.

^{125}I-bFGF-binding Assays

Iodinated bFGF (specific activity = 800–1,200 Ci/mmol) was purchased from New England Nuclear (Boston, MA), aliquoted directly upon arrival, and stored at −70°C. For the cellular binding assays the K562 transfectants were grown for 72 h in a serum-free medium (DME F12) containing 1 g/liter BSA, 8 mg/l transferrin, and 4 mg/l of insulin, or in Ham's F12 medium supplemented with 30 mM $NaClO_3$ (to suppress the sulfation of the GAG chains) and the same additives. Samples of 200,000 cells were incubated for 90 min at 4°C in 200 μl DME F12 supplemented with 1 mg/ml BSA, 25 mM Hepes, pH 7.5, and 10 ng/ml ^{125}I-bFGF, in the absence or presence of 1 μg/ml unlabeled bFGF and with or without 100 ng/ml heparin. The cells were then washed two times with cold PBS and once with 2 M NaCl, 50 mM NaH_2PO_4, pH 7.5. The radioactivities of the salt washes and the cell pellets were counted separately. The values obtained in the presence of 100-fold excess of unlabeled bFGF were considered unspecific binding and were substracted from the total counts. The data are displayed as the means and SDs of three independent experiments.

In the cell-free binding assay, increasing amounts of ^{125}I-bFGF were combined with FGFR1e (6 ng) in the presence or absence of Syn4e (0.8 ng), trypsinized Syn4e (0.8 ng), or heparin (100 ng/ml), in 500 μl of assay buffer (50 mM NaH_2PO_4, pH 7.5, 150 mM NaCl, 2 mg/ml gelatin, and 0.5% Tween 20). Control mixtures consisted of increasing ^{125}I-bFGF or ^{125}I-bFGF and heparin concentrations in assay buffer. All mixtures were supplemented with 20 μl of Ni-NTA resin and incubated on a roller shaker at room temperature for 2 h. Bound label was recovered by centrifugation, washing of the beads in PBS, and discarding of the supernatant. Specific ^{125}I-bFGF binding was measured by substracting the amounts of label bound in control mixtures from the counts associated with the beads in test mixtures. This experiment was carried out three times with two different batches of ^{125}I-bFGF. The data were transformed into concentration equivalents and analyzed as Scatchard plots (Scatchard, 1949) using a computer program for linear curve fitting.

Covalent Cross-linking of ^{125}I-bFGF to FGFR1

Wild-type K562 cells and FGFR1-transfected K562 cells were prepared and incubated with ^{125}I-bFGF, as in the other bFGF-binding studies. After washing with 2 × 1 ml of cold PBS, the cells were incubated with 100 μg/ml (0.27 mM) disuccinimidyl suberate (Pierce, Rockford, IL) in PBS at 15°C for 45 min. The reaction was quenched with 20 mM Tris, pH 7.4, in PBS. The cell samples were boiled for 5 min in 2% SDS, 10% glycerol, 20 mM Tris pH 6.8, 1 mM EDTA, and 0.005% bromophenol blue, and were applied on 6–20% polyacrylamide gradient gels. After running, the gels were stained with Coomassie brilliant blue and dried for autoradiography. Quantitative analysis of the intensity of the bFGF–FGFR1 band was performed with an ImageQuant personal densitometer (Molecular Dynamics, Sunnyvale, CA). Reference bands in the Coomassie-stained gels were also measured to exclude differences in loading.

Immunofluorescence Cytometry

Immunocytofluorometry was performed with a FACSort® (Becton Dickinson & Co., Mountain View, CA), and data were analyzed with the program Lysis II. For indirect immunofluorescence staining, K562 cells were incubated with the designated mouse mAbs at a concentration of 10 μg/ml for 30 min, washed 2×, and then incubated with FITC-labeled goat anti–mouse Ab (Nordic Immunology, El toro, CA). Nonreactive, isotyped-matched mouse mAbs were used to measure background fluorescence. FITC-labeled anti–glycophorin A (GpA) mAb (clone JC159; Dako Glostrup, Denmark) and R-phycoerythrin-labeled anti-CD14 mAb (clone TÜK4; Dako) were incubated together and used at the concentration proposed by the manufacturer. Background was determined with correspondingly labeled isotyped-matched mAbs (Dual Colour Reagent; Dako). The relative mean fluorescence intensity (rMFI) for GpA was calculated as:

$$\text{GpA rMFI} = \frac{MFI_{\text{GpA-treated cells}} - MFI_{\text{background treated cells}}}{MFI_{\text{GpA-untreated cells}} - MFI_{\text{background untreated cells}}}$$

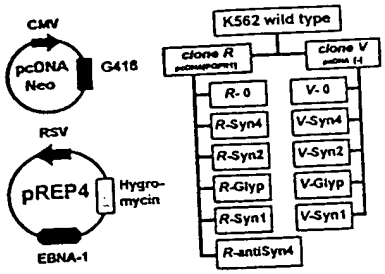


Figure 1. Transfection strategy. K562 cells were first transfected with the integratable vector pcDNA/Neo containing an FGFR1 cDNA or no insert. Two stable subclones, referred to as clone *R* for the FGFR1 transfection and clone *V* for the vector transfection, were then transfected with the episomal vector pREP4, either as such or provided with cDNAs coding for syndecans 4, 2, 1, or glypican (*R*-0, *R*-Syn4, *R*-Syn2, *R*-Glyp, *R*-Syn1, and corresponding *V* cells). In addition, clone *R* was transfected with an antisense syndecan-4 construct (*R*-antiSyn4 cells). The cotransfection was realized using the two different selection markers, G418 for pcDNA/Neo and hygromycin for pREP4.

All experiments were performed at least twice; SEM for all MFI values was <4%.

Results

Cell-surface PG Expression in K562 Cells

The transfection strategy that was adopted to study the FGFR system is illustrated in Fig. 1. Wild-type K562 cells, which do not bind bFGF in specific ways (Partanen et al., 1991), lack any transcriptional message for FGFR1 (Armstrong et al., 1992; our own unpublished data), and express only low levels of cell-surface HS (see below), were first transfected with an integratable pcDNA/Neo vector provided with cDNA for FGFR1(IIIc) or without insert. One stable subclone from the receptor transfection that showed specific binding of ^{125}I-bFGF (further referred to as clone *R*) and one subclone from the control transfection (further referred to as clone *V*) were then further transfected with the episomal vector pREP4, either as such or provided with cDNA inserts coding for syndecans 4, 2, 1, or glypican to enhance the levels of HS in these cells. Clone *R* was also transfected with a syndecan-4 antisense construct, since this syndecan seems to account (at least in part) for the small amounts of endogenous HS expressed by K562 cells. Several approaches were then used to evaluate the effect of these transfections on the expression of HS by K562 cells.

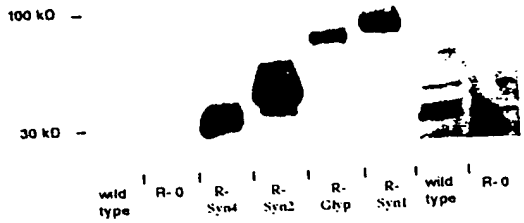


Figure 2. HSPG expression in K562 cells. Triton X-100 extracts of transfected K562, normalized for cell number, were purified by ion exchange chromatography and fractionated by SDS-PAGE after digestion with heparitinase. The Western blot was developed with mAb 3G10, which recognizes the terminal desaturated glucuronate that caps the HS stubs after heparitinase digestion, to detect all proteins substituted with HS, and as an assessment of the number of HS chains that were expressed. Detection was performed by chemiluminescence. Film exposure was given 1 min (*first six lanes*) or 5 min (*last two lanes*). PG-transfected K562 populations expressed high numbers of HS chains, which were readily detected after 1 min of exposure, on a single core protein. HS expression was barely detectable in wild-type and *R*-0 cells after 1 min, but bands of 120 kD, 50 kD, and mostly 35 kD were visible after the longer exposure. The stronger 35-kD band in these cells reacted with the syndecan-4–specific mAb 8G3 (not shown).

After heparitinase digestion, any protein that is substituted with HS can be traced by mAb 3G10, since this antibody recognizes the Δ-glucuronate that caps the HS stubs (David et al., 1992*a*). In Western blots of PG extracts, this antibody detected several weak bands in wild-type and in *R*-0 cells (mainly ~35-kD bands visible after more prolonged exposures), and strong ~35-, 48-, 64-, and 85-kD bands in the *R*-Syn4, *R*-Syn2, *R*-Glyp, and *R*-Syn1 transfectants, respectively (Fig. 2). These proteins were positively identified as the expected transfectant proteins with the core protein–specific mAbs 8G3 (syndecan-4), 10H4/6G12 (syndecan-2), S1 (glypican), and 2E9 (syndecan-1) (not shown).

Analysis of the amount of HS expressed at the surface of the transfectants, by quantitative immunofluorescence flow cytometry using the HS-specific mAb 10E4 (David et al., 1992*a*), revealed marked (5–10-fold) increases in cell-surface HS in all *R*-PG transfected cell populations (Fig. 3, *a* and *b*). The expression of the 10E4 epitope at the surface of *R* antisense transfectants, in contrast, was reduced by ~50% in comparison with *R*-0 cells (Fig. 3 *c*). Similar analyses with protein-specific antibodies confirmed the cell-surface expression of the transfectant PGs in transfectant cells, the cell-surface expression of endogenous syndecan-4 in wild-type cells, a >10-fold increase of the cell-surface expression of the syndecan-4 core protein in *R*-Syn4 cells, and the decrease (by 80%) of the cell-surface expression of this syndecan in the syndecan-4 antisense transfectants (not shown). Very similar PG expressions were also achieved in *V*-PG cotransfection experiments (data not shown).

All *R* transfectants were also metabolically labeled with [^{35}S]sulfate for 24 h. PG was extracted from the cells with Triton X-100, and then further purified by ion exchange chromatography on DEAE and MonoQ, as shown for the *R*-0 and the *R*-Syn4 transfectants in Fig. 4 *a*. Extracts from *R*-PG cells yielded two to fourfold more label per cell than the *R*-0 cell extract. The amount of [^{35}S]syndecan-4 recovered by immunoprecipitation from *R*-Syn1, *R*-Syn2, or *R*-Glyp extracts, in contrast, was identical or slightly lower than the amount of [^{35}S]syndecan-4 recovered from *R*-0 cells (not shown). Both the *R*-PG and *R*-0 materials eluted as a broad early peak (0.45–0.65 M; peak A) and a more distinct later peak (0.70–0.85 M, peak B). All PG transfections lead to increases in both peak A and B materials, but the A/B peak ratio was always higher in *R*-PG extracts than in the *R*-0 extract. Qualitatively similar elution profiles were obtained for immunopurified PG (not shown). Endogenous syndecan-4 immunopurified from *R*-0 cells mimicked the profile obtained for the total PG extract

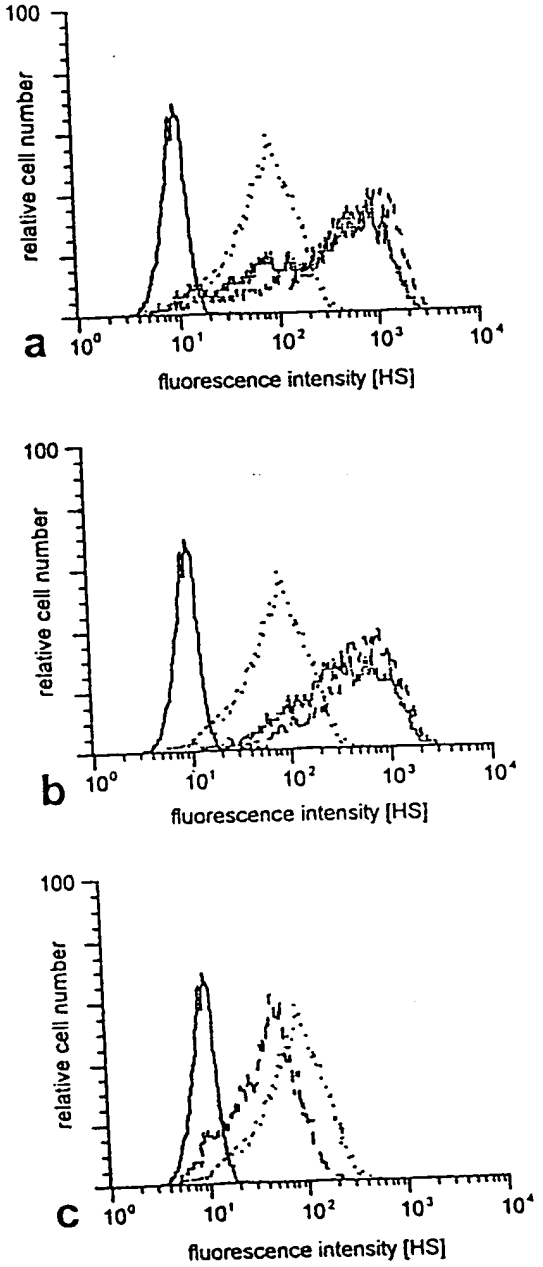


Figure 3. Cell-surface expression of HS in K562 cells. Cell-surface HS was measured by quantitative immunofluorescence cytometry using the HS-specific mAb 10E4. The background fluorescent signal (*solid line*) and the cell-surface HS expression in *R*-0 cells (*dotted line*), *R*-Syn1 cells (*narrow dotted line*), and *R*-Syn2 cells (*dashed line*). (*b*) The background fluorescence and the cell-surface HS expression in *R*-0 cells, *R*-Glyp (*narrow dotted line*), and *R*-Syn4 (*dashed line*) cells. (*c*) The background fluorescence and cell-surface HS expression in *R*-0 cells (*dotted line*) and *R*-antiSyn4 cells (*dashed line*).

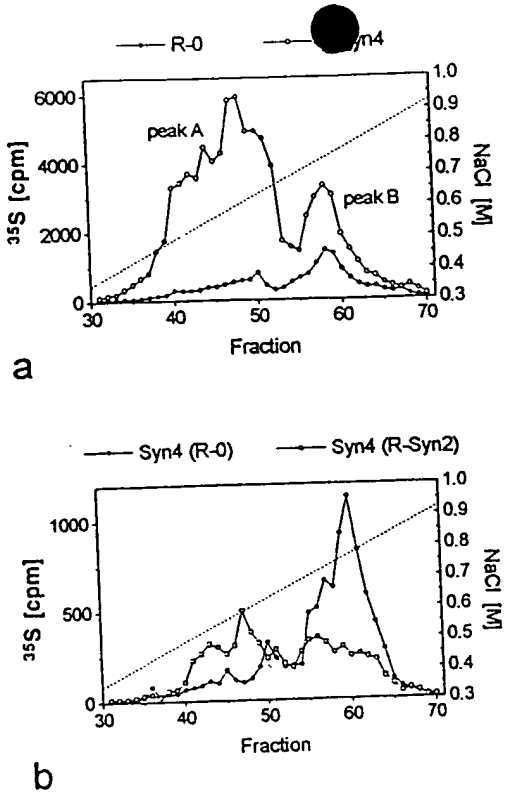


Figure 4. Ion exchange chromatography of the K562 PGs. ^{35}S-labeled PGs produced by the various transfectants were extracted with detergent and subjected to ion exchange chromatography. (*a*) The MonoQ elution profiles obtained for the total extracts from the *R*-0 and the *R*-Syn4 transfectant. The elution profiles obtained for the other PG transfectants were qualitatively similar to the one obtained for *R*-Syn4. The profile obtained for the *R*–anti-Syn4 transfectant was similar to that obtained for *R*-0 cells. (*b*) The elution profiles obtained for the endogenous syndecan-4 immunopurified from *R*-0 and *R*-Syn2 cells after digestion with chondroitinase ABC to remove the CS-substituted forms.

from these cells (prominent B peak), whereas the recombinant PGs and also the endogenous syndecan-4 immunopurified from *R*-PG cells eluted like total *R*-PG extracts (more prominent A peak).

Early (A peak) and late (B peak) eluting materials from total extracts were collected as separate pools and used for the further immunopurification of endogenous and/or transfectant PG on the corresponding antibody. Similar pools were also made for the eluted immunopurified PGs. Analysis of the GAG chain compositions of these immunopurified PGs revealed that the syndecans 1, 2, and 4 isolated from the corresponding transfections contained [^{35}S]HS as well as [^{35}S]CS. This was observed for both A and B peak–derived PGs (with a tendency for a higher HS content in B peak than in A peak materials, 40–60% versus 20–50%). This was also the case for the endogenous syndecan-4 expressed by *R*-0 cells. Glypican isolated from either the A or the B peak of the *R*-Glyp extract, on the other hand, carried almost exclusively HS (>90%). The fractionation of intact, heparitinase-, chondroitinase-, and doubly digested immunopurified PG samples by SDS-PAGE analysis and Western blotting indicated that in all instances (except for syndecan-1), the HS and CS chains were present on separate coreprotein populations, with little evidence for hybrid PG (shown for syndecan-4 from *R*-Syn4 extracts in Fig. 5).

Gel filtration chromatography indicated that the sizes of the protein-free HS chains were nearly invariant (~14 kD), whether isolated from different immunopurified PGs or from peak A or B materials (not shown). Ion exchange chromatography indicated that protein-free HS chains derived from A peaks were less anionic than chains derived

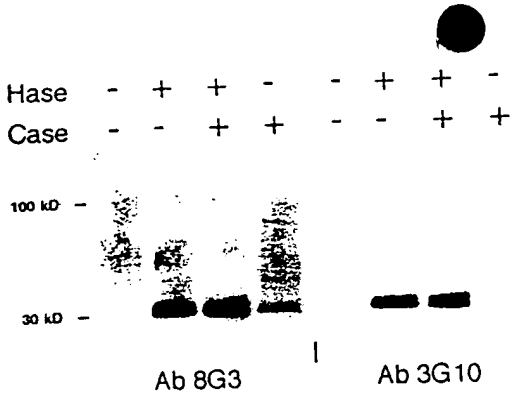


Figure 5. GAG chain composition of the K562 PGs. Immunopurified syndecan-4 derived from the corresponding transfectant (*R*-Syn4) was left untreated (−) or subjected (+) to heparitinase (*Hase*), chondroitinase ABC (*Case*), or both enzymes. The digests were fractionated by SDS-PAGE, blotted, and incubated with the syndecan-4 core protein–specific mAb 8G3 or the anti-Δ-HS mAb 3G10. Comparison of the banding patterns after combined and single enzyme digestions indicated that the majority of syndecan-4 molecules were substituted with HS and a smaller proportion were substituted with CS, with little or no evidence for hybrid molecules. Similar results were obtained for the syndecan-2. Syndecan-1 materials contained higher amounts of CS, in part as true hybrids. Glypican carried almost exclusively HS chains.

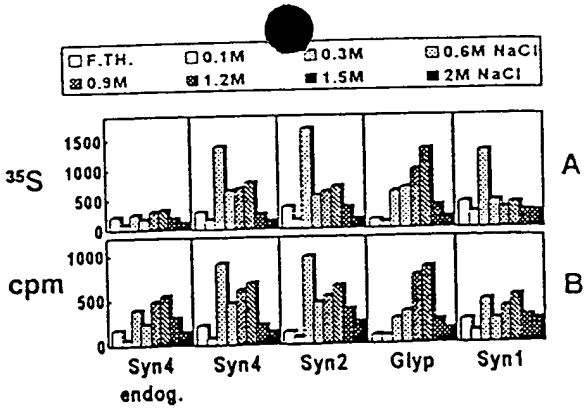


Figure 6. K562 PG binding to immobilized bFGF. Immunopurified, ^{35}S-labeled PGs isolated from peak A and peak B fractions (see Fig. 4) were applied to a bFGF column and eluted with a salt step gradient (up to 2 M NaCl). Glypican eluted as nearly one peak (at 1.2 M), the syndecans eluted as two major peaks, one at 0.3 M and a second at 1.2 M NaCl. The low salt eluates (fall through, 0.1, 0.3, and 0.6 M) contained ~70% CS, and the high salt eluates (0.9, 1.2, 1.5, and 2 M) contained ~90% HS.

from B peaks, but no differences in charge density were observed between HS chains from corresponding peaks derived from different PGs (not shown). Finally, sizing of the different immunopurified cell-surface PGs by SDS-PAGE (after a treatment with chondroitinase ABC to remove the CS-substituted forms) indicated that the more anionic forms of each HSPG species (B peak) were significantly more retarded than the less anionic forms (A peak). Yet, after heparitinase, A and B peak PGs yielded core proteins of similar sizes, indicating increasing numbers of HS-side chains per core protein in the more anionic PGs (not shown).

In another series of experiments, the ^{35}S-labeled *R*-0 and *R*-PG transfected cells were surface biotinylated immediately before the detergent extraction, subjected to ion exchange chromatography, and PG was immunopurified as described above. Early (A peak) and late (B peak) eluting fractions of each PG were then incubated with streptavidin beads to isolate the surface-exposed forms of these PGs. The percentage of streptavidin-bound label did not differ among A and B fractions, indicating that the various forms of a particular PG were equally well represented on the cell surface. This percentage ranged from 60 to 80% for syndecans or glypican isolated from the corresponding PG-transfected cells, but was only ~30% for syndecan-4 isolated from *R*-0 cells (data not shown). From the total label, the HS content, and the size of the biotinylated fraction, it was calculated that the PG transfections resulted in five- to sevenfold increases in cell surface [^{35}S]HSPG.

Altogether, these data demonstrated that overexpression of various cell-surface PGs in K562 cells lead to marked enhancements in cell-surface HS expression. This enhancement was most pronounced for the lesser sulfated forms of this glycosaminoglycan that were present on PGs of low chain valency, and it occurred at the detriment of the glycanation of the endogenous cell-surface PG (lower average HS sulfation and chain valency). The latter was confirmed by the immunopurification of endogenous syndecan-4 from *R*-0 and *R*-PG cells, digestion of the PG with chondroitinase ABC, and analysis of the HS-substituted syndecan-4 by ion exchange chromatography over MonoQ (Fig. 4 *b*). These findings indicated that the effects of the transfections on HS and HSPG synthesis were not simply additive, but also competitive, somewhat analogous to the effect of β-xylosides on the synthesis of CS (stimulation) and CSPG (inhibition) by cells. They also underscored the conclusion that the gain in cell-surface HS in the transfectants is driven by the transfectant PG.

Cell-surface PGs from K562 Cells Bind bFGF

To evaluate the bFGF-binding properties of the cell surface PGs, the various forms were immunopurified from the corresponding *R*-PG transfectants and allowed to bind to biologically active recombinant bFGF that was immobilized on Ni-NTA agarose via an aminoterminal 6xHis-tag. After equilibration, the column was eluted with an NaCl step gradient (Fig. 6). Syndecans (isolated from A or B peaks) eluted as two major peaks, one at 0.3 M and a second at 1.2 M NaCl. Analysis of the GAG compositions of the eluted syndecan fractions indicated that the pool of the first four fractions (nonbound, and eluting ≤0.6 M NaCl) contained mainly CS (~70%), whereas the pool of the four last fractions (eluting ≥0.9 M) contained almost exclusively HS chains (~90%). Glypican, which contained only HS, eluted as nearly one peak at 1.2 M NaCl. These data indicate that only HS-carrying forms of the PGs bind significantly to bFGF, and they confirm that most syndecan cores expressed in K562 cells display either HS or CS chains rather than a combination of both.

Heparin Sensitivity of the Binding of bFGF to FGFR1 in K562 Cells

We then measured the binding of ^{125}I-bFGF to PG- and

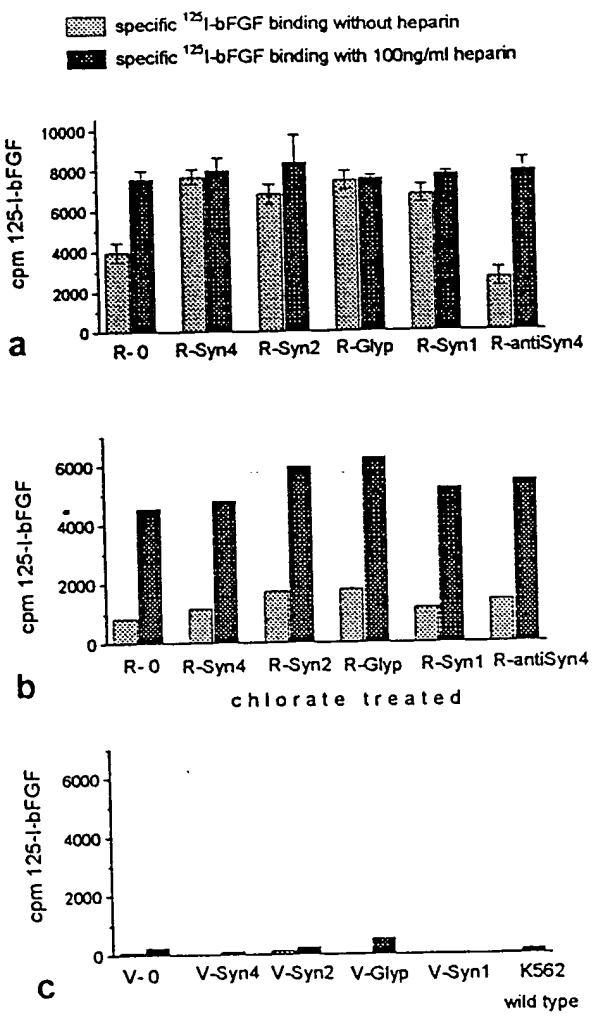


Figure 7. Binding of bFGF to FGFR1 in K562 cells. Aliquots, of 200,000 cells each, were incubated with 10 ng/ml of ^{125}I-bFGF for 90 min at 4°C in the absence or presence of 1 μg/ml of unlabeled bFGF and with or without 100 ng/ml of heparin. The bars indicate the amounts of iodinated bFGF that remained specifically bound after a neutral 2-M NaCl wash of the cells. Results are shown for receptor-transfected cells (*a*), chlorate-treated, receptor-transfectant cells (*b*), and for non-receptor-transfected cells (*c*). The values for receptor-transfectant cells are given as the means and SDs of three independent experiments.

non-PG-transfected *V* and *R* cells, as well as the effect of exogenous heparin on this binding (see Materials and Methods). Compared to non-PG transfectants, the *V*-PG and the *R*-PG transfectants showed similar (near 10-fold) increases in label in the neutral salt washes of the cells, and this label could barely be displaced by a 100-fold excess of unlabeled bFGF (not shown). All *R*-PG transfectants showed an increase in specific salt-resistant binding of ^{125}I-bFGF when compared with the *R*-0 and the *R*-antiSyn4 transfectants (Fig. 7 *a*). Adding soluble heparin at a concentration of 100 ng/ml doubled specific bFGF binding to RO cells and tripled specific bFGF binding to R-antiSyn4 cells, but had no effect on specific bFGF binding by the *R*-PG transfectant cells (Fig. 7 *a*). The specific bFGF binding in the presence of heparin was roughly constant for all *R* cell populations (excluding differences in the number of FGFR1 receptors per cell among the various transfectants), and was calculated to correspond to ~30,000 binding sites per cell. Chlorate treatment of all *R* transfectant cell populations resulted in a decrease of the specific binding to 15–25% of the value obtained in the presence of 100 ng/ml of heparin (Fig. 7 *b*). Neither wild-type K562 cells nor any of the PG transfectants of the *V* clone revealed significant levels of specific ^{125}I-bFGF binding, demonstrating that the assay was measuring FGFR1-related bFGF binding only (Fig. 7 *c*).

Heparin Sensitivity of the Affinity Cross-linking of bFGF to FGFR1 in K562 Cells

The participation of the cell-surface HS in the bFGF–receptor interaction was also investigated by affinity cross-linking experiments. Covalent cross-linking of ^{125}I-bFGF to the various *R*-transfectants demonstrated a putative bFGF–FGFR1 complex with an apparent molecular mass of ~140 kD. The formation of this labeled complex was inhibited by adding an excess of cold bFGF, and it did not occur in wild-type K562 cells. Quantitative densitometric analysis of the bFGF–FGFR1 complexes in the various transfectants, formed in the presence and in the absence of exogenous heparin, gave the following results: 100 ng/ml of heparin *increased* the yield of labeled bFGFR cross-links by 26% for *R*-0, by 40% for *R*-antiSyn4, and eightfold for chlorate-treated *R*-0 cells; the same heparin concentration *decreased* ligand cross-linking by 33% for *R*-Syn4, 46% for *R*-Syn2, 58% for *R*-Glyp, and 23% for *R*-Syn1 (Fig. 8). Increased yields of specific growth factor–receptor complexes in non-PG transfectants and sulfate-starved cells when heparin was added, were consistent with the results from the binding experiments that had revealed an enhancement of the bFGF–FGFR1 interaction by heparin in these cells (Fig. 7). Negative effects of heparin on the yield of growth factor–receptor cross-links in PG transfectants, where heparin did not affect the extent of the specific binding of the growth factor (Fig. 7), suggested modal differences between heparin- and PG-mediated specific bFGF–FGFR1 interactions.

FGFR1 and HS Dependency of the bFGF-induced Block in Erythroid Differentiation of K562 Cells

K562 cells are multipotential malignant hematopoietic cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic, and monocytic series. A treatment with hemin or the tyrosine kinase inhibitor herbimycin A reduces the intracellular tyrosine phosphorylation in K562 cells and stimulates their erythroid differentiation (Richardson et al., 1987; Honma et al., 1989). Exposure of K562 cells to 10^{-9} M PMA, in contrast, results in a reduced expression of erythroid-specific proteins, along with a weak myelomonocytic induction (Papayannopoulou et al., 1983). Erythroid differentiation of the K562 transfectants in the presence of growth factor was therefore measured as a test for the functionality of the FGFR1 and to evaluate the possible contributions of cell-surface PG in receptor-mediated growth factor effects.

For these experiments, PG- and receptor-transfected K562 cells were grown in a defined serum-free medium (see Materials and Methods). After 72 h of growth under these conditions, bFGF was added in concentrations of

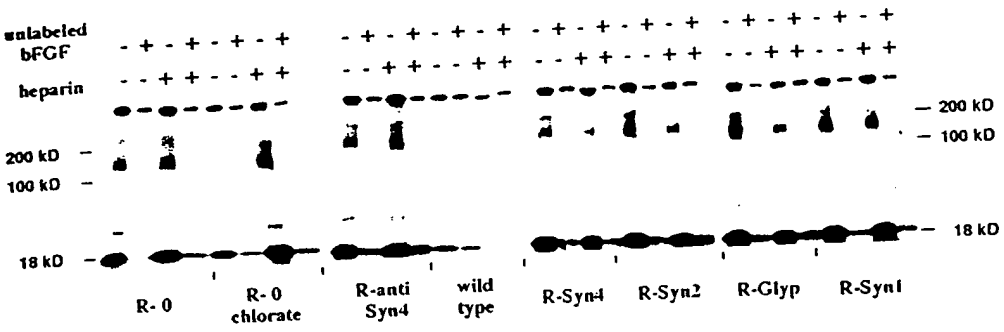


Figure 8. Cross-linking of bFGF to FGFR1 in K562 cells. For affinity cross-linking, the various cell populations were incubated with ^{125}I-bFGF, following the same procedures as described in the legend to Fig. 7. After washing off free label, cell-bound bFGF was cross-linked with 0.27 mM freshly dissolved DSS. The cells were then boiled in SDS buffer and fractionated by SDS-PAGE. After autoradiography, the intensities of the ~140-kD bFGF–FGFR1 bands were measured with a densitometer. 100-fold excess of unlabeled bFGF eliminated the formation of a labeled bFGF–FGFR1 complex in all receptor-transfected cells. Heparin potentiated receptor cross-linking in non–PG-transfected populations (by 26% in *R*-0 cells, by 40% for *R*-antiSyn4 cells) and most strikingly in chlorate-treated *R*-0 cells (eightfold increase). Wild-type K562 cells lacked any specific receptor cross-linked band. Heparin, on the other hand, decreased the cross-linking of ligand to the receptor in the PG-transfected cell populations (by 33% for *R*-Syn4, 46% for *R*-Syn2, 58% for *R*-Glyp, and 23% for *R*-Syn1).

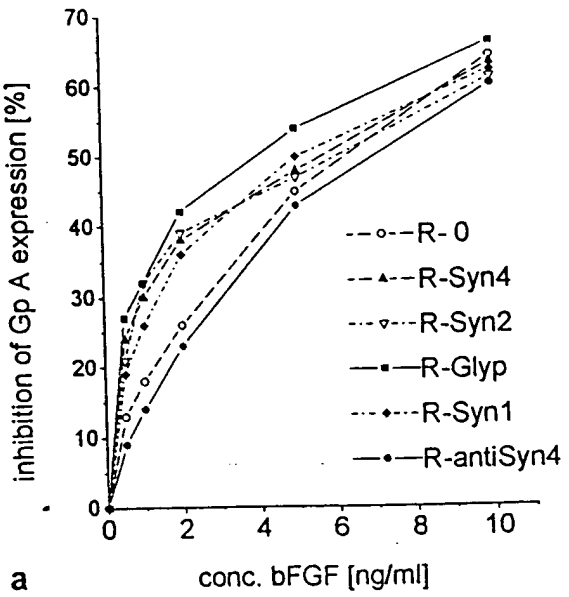
0.5–10 ng/ml, and 72 h later, the GpA and CD14 expressions were measured by immunofluorescence flow cytometry. A dose-dependent suppression of GpA was obtained in all these cells (Fig. 9 *a*). At the high concentration of 10 ng/ml of bFGF, the mean GpA level in all *R* transfectants was suppressed to approximately one third of the control value (without bFGF), but at lower bFGF concentrations, the PG-transfected *R* cells were more responsive than the *R*-0 and the *R*-antiSyn4 cells. At a bFGF concentration of 10 ng/ml, the CD14 expression was increased by ~50% for all six *R* transfectants (data not shown). When treated with chlorate, the same cell populations were nearly unresponsive to bFGF, but the effect of bFGF on the GpA expression could largely be restored by the addition of heparin (Fig. 9 *b*). Neither wild-type K562 cells nor any *V* transfectant showed a change in GpA or CD14 expression when exposed to bFGF, with or without heparin (Fig. 9 *c*). Yet, these cells and the *R* cell populations showed similar decreases in GpA expression in response to 10^{-9} M PMA after 72 h (shown only for wild-type cells in Fig. 9 *c*). The interpretation that stimulation of FGFR1 increased intracellular tyrosine phosphorylation and consequently blocked erythroid differentiation was supported by the reverting effect of tyrosine kinase inhibitors. In *R* cells that were preincubated with 30 μM of genistein for 2 h before the addition of 10 ng/ml of bFGF, the GpA and CD14 expressions remained largely unchanged (data not shown).

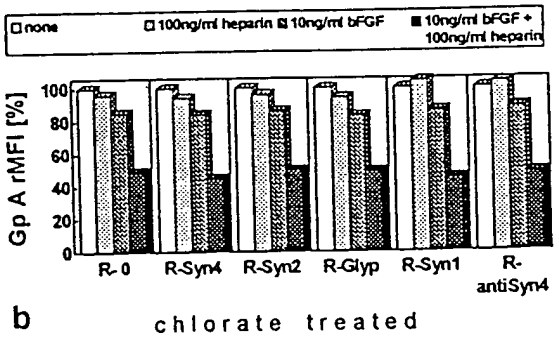
Binding of bFGF to Surface-bound FGFR1 and HSPG Ectodomains

Finally, to exclude possible contributions by non transfectant PGs or other membrane-anchored molecules, we also measured the effect of HSPG on the binding of bFGF to its receptor under cell-free conditions. In this assay, we used recombinant FGFR1e and Syn4e provided with COOH-terminal 6xHis tags that bind with high affinity (K_d = 10^{-13}) (Hochuli et al., 1987) to Ni-loaded beads (Fig. 10 *a*). The affinity of bFGF for the ectodomains was calculated from the label coprecipitated with the Ni-NTA beads versus the free label at various bFGF concentrations (Fig. 10 *b*). The dissociation constant for the interaction of bFGF with Syn4e in the absence of FGFR1e was 2.7 nM (not shown). The calculated dissociation constant for the direct bFGF–FGFR1e interaction in the absence of any source of HS in this assay was 1.8 nM, threefold higher than the dissociation constant for the interaction of bFGF with the combination of FGFR1e and Syn4e (0.6 nM) or the combination of FGFR1e and chondroitinase ABC–treated Syn4e (not shown). In contrast, the affinity of bFGF for the combination of FGFR1e and heparitinase-treated Syn4e was identical to its affinity for FGFR1e. The addition of soluble heparin (100 ng/ml) to bFGF slightly increased the affinity of the growth factor for FGFR1e (K_d = 1.1 nM), whereas trypsin-treated Syn4e added at similar concentrations as Syn4e had no effect on the binding (K_d = 1.7 nM). For the combination of FGFR1e and Syn4e, the concentrations of the ectodomains were chosen such that the maximal number of bFGF-binding sites contributed by each component were individually similar. Yet the maximal number of binding sites obtained for the combination of FGFR1e and Syn4e did not differ from the maximal number of binding sites obtained for these ectodomains tested individually. This suggested a simultaneous binding of bFGF to both ectodomains, as a ternary complex that has greater stability than that mediated by soluble heparin.

Discussion

Our results demonstrate that three different syndecans and glypican can promote the binding and activation of a specific kinase receptor form, i.e., the IIIc splice variant of the FGFR1, by a specific member of the FGF family, i.e., bFGF (FGF2), when expressed with the FGFR1 as coreceptor pairs in transfectant K562 cells. All the forms that were tested boost the expression of cell-surface HS in these hematopoietic cells, facilitating the saturation of the receptor with growth factor and increasing the sensitivity of the cells to low doses of the growth factor that inhibit their erythroid differentiation. We conclude that cell-surface PGs can function as partners for the tyrosine kinases in a dual FGFR system, and that several different forms of this category of cell-surface components can provide the source of HS that is required for effective FGF–FGFR bind-





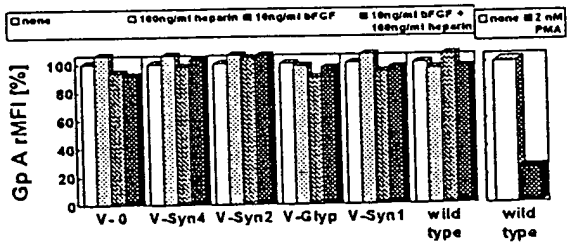


Figure 9. Effect of bFGF on GpA expression in K562 cells. The various K562 cell populations were cultured for 72 h in serum-free medium supplemented with BSA, transferrin, and insulin. After exposure to the indicated concentrations of bFGF for an other 72 h in this medium, the cell-surface GpA expression was measured by quantitative immunofluorocytometry. The displayed relative mean fluorescence intensity values were calculated as indicated in Materials and Methods. Receptor-transfected cell populations (*a*) responded with a dose-dependent decrease in GpA expression. At the maximal concentration of 10 ng/ml of bFGF, the GpA was reduced by 60–70% in all transfectants. PG-transfected clones, however, responded at significantly lower bFGF concentrations than *R*-0 and *R*-anti-Syn4 cells, e.g., at bFGF concentrations as low as 0.5 ng/ml *R*-Glyp still showed a 27% suppression, and *R*–anti-Syn4 cells showed only a 9% suppression. Chlorate-treated *R* cells (*b*) were nearly unresponsive to bFGF, but the effect of bFGF could be restored with exogenous heparin (added at 100 ng/ml). Exposure of the wild-type or the non–receptor-transfected cell populations (*c*) to bFGF in combination with or without heparin did not result in significant changes in GpA mean fluorescence intensity. Treatment of K562 cells with the phorbol ester PMA (2 nM, over 72 h) induced an 80% loss of GpA expression (shown only for wild-type cells in *c*).

ing interactions. A low incidence of active HS sequences in these PGs may be compensated by their membrane anchorage and concentration at the cell surface.

K562 Cells as a Model for Studying Cell-surface HS

K562 cells were selected for these studies because a survey of a large panel of cells with the HS-specific mAbs 10E4 and 3G10 had indicated that these cells were able to synthesize authentic HS, a minimal requirement to potentially support bFGF–receptor interactions, but in low and possibly insufficient amounts to support these interactions efficiently. The aim was to test whether transfections with cDNAs coding for cell-surface PGs could compensate for this relative HS deficiency. The results show that after these transfections, K562 cells are capable of expressing ~5–10-fold higher levels of cell-surface HS, and that the endogenous and transfectant cell-surface PGs that account for this HS can be fractionated in distinctive charge and size classes that result from the intrinsic variability of the posttranslational modifications of these proteins. Comparative quantitative immunocytofluorometry indicated that the HS expression in the K562 transfectants reached similar levels as in human lung fibroblasts (not shown), suggesting that these transfectants provide relevant models for the display of cell-surface HS in constitutive high expressors. It may be significant, however, that the gain of HS in these cells is more pronounced for the PG fractions that elute early from MonoQ (substituted with fewer and less sulfated chains) than for those that elute later in the salt gradient (substituted with more and more highly sulfated chains) (Fig. 4). Together with the reduced levels of HS glycanation of the endogenous syndecan-4 in the transfectants, these results suggest that in K562 cells, individual core proteins compete with each other for a limiting HS glycanation machinery, and that in high expressors, a smaller proportion of the PGs therefore reaches the most extensive levels of substitution and modification. These findings are reminiscent of results obtained for the synthesis of antithrombin III–binding HS sequences in transfectant endothelial and fibroblastic cells, where several consecutive transductions of a syndecan-4 expression vector progressively enhanced the production of core protein and total HS in these cells, but reduced the levels of antithrombin III–binding HS present on transfectant and endogenous PG (Shworak et al., 1994). This suggests that the production of defined HS sequences can be saturated and that the specific activities of the PGs in terms of these sequences depend at least in part on the core protein expression levels. In the K562 PG transfectants, the transfectant cores drive the synthesis of ~90% of the cell-surface HS, but these expression levels still appear compatible with the production of fully modified forms of PG and the produc-

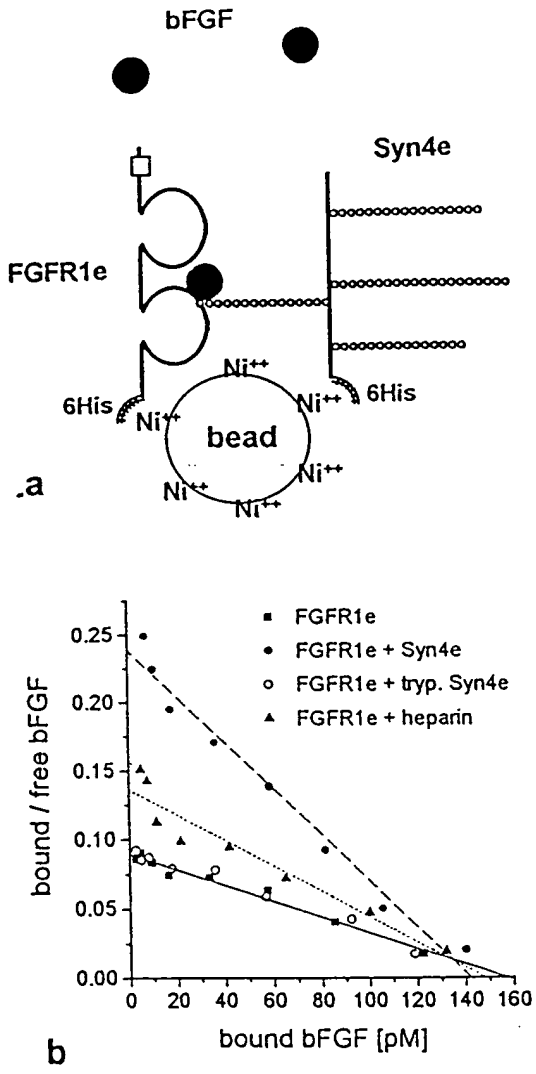


Figure 10. Coimmobilized HSPG increases the affinity of bFGF for the ectodomain of FGFR1. Plasmids coding for COOH-terminal 6xHis-tailed forms of the ectodomain of FGFR1 (*FGFR1e*) and syndecan-4 (*Syn4e*) were expressed in K562 cells. These 6xHis tags bind with high affinity ($K_d = 10^{-13}$) to Ni-loaded beads (*a*). Binding of radiolabeled bFGF to Ni-NTA–immobilized FGFR1e in the presence or absence of Syn4e, trypsinized Syn4e (which lacks the 6xHis tag and therefore can not bind to the Ni-NTA resin), or heparin was measured at varying concentrations of growth factor (*b*). The affinity of the binding was calculated from the specifically bound versus free radiolabel at various bFGF concentrations. The data are depicted as Scatchard plots. The concentrations of FGFR1e and Syn4e used were such that the maximal number of bFGF-binding sites provided by each component individually (as determined from separate binding experiments) were identical.

tion of the sequences required for bFGF binding and activation. It is conceivable, however, that similar transfections in cells that express large near-saturating amounts of endogenous HSPG could have adverse effects on the synthesis or colinearity of the sequences that are required for bFGF activation, and that this competition might explain how the expression of syndecan-1 in 3T3 cells suppresses the growth response of these cells to bFGF (Mali et al., 1993).

Facilitation of bFGF–Receptor Binding by Cell-surface HS

The effects of chlorate on the binding of bFGF to *R* transfectants and of heparin on chlorate-treated *R* transfectants were consistent with the observations of several other investigators, suggesting a clear HS-dependency of the specific binding of bFGF to FGFR1(IIIc) and indicating that K562 cells were able to produce the HS sequences that are required for the stimulation of this binding. Heparin, however, also enhanced the levels of specific bFGF binding in *R*-0 cells that were not treated with chlorate, indicating lack of receptor saturation in these cells despite normally saturating concentrations of added growth factor, possibly caused by PG receptor imbalances in these receptor-overexpressing cells. The saturation of the receptor in the *R*-PG transfectants confirms this interpretation and indicates that all the different cell-surface PGs tested can complement for the relative HS deficiency of these cells. Reduced levels of receptor saturation in *R*–anti–syndecan-4 transfectants in comparison to *R*-0 cells support the contention that cell-surface PGs contribute to receptor binding in K562 cells, in apparent discrepancy with previous suggestions that these forms are inactive or even inhibitory in this respect (Aviezer et al., 1994*a*). Distinctive PG requirements for activation of the receptor in *cis*- and *trans*-modes, or unique activities of the PGs in these cells, could account for this discrepancy.

Our cell-free assay demonstrates that bFGF binds to the ectodomain of the two-Ig domain form of human FGFR1 in the absence of heparin, and that heparin moderately enhances the affinity of this binding interaction, which agrees with the results reported by several other investigators with similar constructs (Kiefer et al., 1991; Bergonzoni et al., 1992; Roghani et al., 1994). In this assay, a syndecan ectodomain made by K562 cells that could be coimmobilized with receptor proved to be an effective strengthener of the binding interaction, whereas the same ectodomain provided in equimolar amounts, but in soluble form, had no detectable activity. The failure of these soluble ectodomains is in agreement with previous binding results obtained for receptor–reporter fusion proteins and soluble cell-surface PG in cell-free assays (Aviezer et al., 1994*a*) and for the activation of receptor in HS-deficient cells by exogenously added PG (Aviezer et al., 1994*b*), whereby several of the cell-surface PGs that were studied here were proven to be ineffective. All together, these findings suggest that cell-surface PGs are not intrinsically ineffective, but that membrane-imbedded and solubilized forms of the PGs from a particular cell differ in their activities on bFGF–receptor binding in that the former lead to higher effective concentrations of reactants with higher apparent binding affinities as a result.

The results from the affinity cross-linking experiments are also consistent with a role for cell-surface HS in the receptor–ligand interaction. They show specific receptor binding in *R* cells and an increase in receptor–cross-linked ^{125}I-bFGF for the *R*-0 and chlorate-treated *R*-PG cell populations upon the addition of heparin, consistent with the stimulatory effect of heparin on receptor occupancy in these cells. Somewhat surprisingly, since heparin did not promote or decrease receptor occupancy in *R*-PG trans-

fectants, heparin very consistently decreased the cross-linking efficiency in all *R*-PG cells. This difference in receptor–ligand cross-linking efficiency between PG-mediated and (in the presence of a large excess of heparin) probably heparin-mediated receptor–ligand complexes suggests the formation of distinctive receptor–ligand complexes in the two situations. Cross-linking likely involves sites within the bFGF–receptor complex other than those directly involved in the binding interaction, and depends on the configuration of the complex, the realized approximations, and the stability of the complex. Conformational changes induced by exogenous heparin, but not by HS, that may be irrelevant for binding might disturb bFGF cross-linking. The finding that ternary complexes mediated by surface-immobilized ectodomains are more stable than heparin-mediated complexes might also be relevant and relate to the reduction in FGFR–FGF cross-links in the presence of heparin. The observation at least suggests that exogenously added heparin does not exactly reproduce the process of PG-mediated binding and cannot be used as the sole model to define the molecular requirements for receptor occupancy by growth factor and activation.

Receptor Activation by Cell-surface HS

The fact that K562 cells that expressed high levels of cell-surface HS, (*R*-PG cells) responded more clearly to low bFGF concentrations than low expressors, (*R*-0 and *R*-anti-Syn4 cells) further supports the contention that, at low doses of growth factor, ligand-induced FGFR stimulation and signaling depend on the availability of sufficient and appropriate sources of HS at the cell surface (Roghani et al., 1994). Our data show that several different cell-surface PGs originating from separate molecular families are able to provide this source, at least for the bFGF–FGFR1 interaction, in cells with the appropriate HS-synthesizing machinery, and when expressed at the cell surface of the receptor-expressing cells. Relatively low specific activities of the cell surface PGs with respect to the fostering of receptor–growth factor interactions may be compensated by this membrane association, essentially limiting the activity of these PGs to the *cis* mode. Considering the relative ineffectiveness of the soluble PG ectodomains as promoters of the bFGF–receptor interaction, it may be significant that all syndecans have conserved a putative protease cleavage site in their ectodomain, and that glypicans are linked to the cell surface by phospholipase-susceptible bonds. Syndecan shedding is known to occur, at least under in vitro conditions, and phospholipase D activities that release soluble bFGF–HSPG complexes have recently been identified in HeLa cell and bone marrow stromal cell cultures (Metz et al., 1994). From our results, we would predict that at low concentrations of growth factor, protease- and lipase-induced sheddings of the cell-surface PGs will lead to a dilution of the reactants, dissociation of the receptor complexes, and downregulation of the signaling pathway, unless other PGs with possibly unique *trans*-activation potentials, such as the perlecan synthesized by cultured fetal lung fibroblasts (Aviezer et al., 1994*b*), can compensate for this loss. This leads to the speculation that PG shedding may provide means for acute regulation of *cis*-activated heparin-dependent pathways, next to possibly slower regulations via controls on the synthesis of the core proteins and the required HS sequences.

The authors are particularly grateful to Helga Ceulemans, Christien Coomans, and An Rayé for their expert technical assistance, and to Bart de Strooper, Mathijs Baens, Luc van Rompaey, and Peter Marynen for many helpful suggestions. We also thank the staff of the Laboratories for Experimental Immunology and Experimental Hematology for advice on the use of the FACSort and the Lysis II program.

This work was supported by grants 3.0073.91 and 1.5.418.93 from the Nationaal Fonds voor Wetenschappelijk Onderzoek of Belgium, by grant 7.0033.92 from the action Levenslijn-Multiple Sclerose, by a grant from the Belgian Cancer Association, and by the Interuniversitary Network for Fundamental Research sponsored by the Belgian government (1991–1995). G. David is a research director of the Nationaal Fonds voor Wetenschappelijk Onderzoek of Belgium.

Received for publication 10 February 1995 and in revised form 22 November 1995.

References

Armstrong, E., S. Vainikka, J. Partanen, J. Korhonen, and R. Alitalo. 1992. Expression of fibroblast growth factor receptors in human leukemia cells. *Cancer Res.* 52:2004–2007.

Aviezer, D., E. Levy, M. Safran, C. Svahn, E. Buddecke, A. Schmidt, G. David, I. Vlodavsky, and A. Yayon. 1994*a*. Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor. *J. Biol. Chem.* 269:114–121.

Aviezer, D., D. Hecht, M. Safran, M. Eisinger, G. David, and A. Yayon. 1994*b*. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. *Cell.* 79:1005–1013.

Bergonzoni, L., P. Caccia, O. Cletini, P. Sarmientos, and A. Isacchi. 1992. Characterization of a biologically active extracellular domain of fibroblast growth factor receptor 1 expressed in *Escherichia coli*. *Eur. J. Biochem.* 210:823–829.

Chellaiah, A.T., D.G. McEwen, S. Werner, J. Xu, and D.M. Ornitz. 1994. Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. *J. Biol. Chem.* 269:11620–11627.

David, G., V. Lories, B. Decock, P. Marynen, J.-J. Cassiman, and H. van den Berghe. 1990. Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulfate proteoglycan from human lung fibroblasts. *J. Cell Biol.* 111:3165–3176.

David, G., X.M. Bai, B. van der Schueren, J.-J. Cassiman, and H. van den Berghe. 1992*a*. Developmental changes in heparan sulfate expression: in situ detection with monoclonal antibodies. *J. Cell Biol.* 119:961–975.

David, G., B. van der Schueren, P. Marynen, J.-J. Cassiman, and H. van den Berghe. 1992*b*. Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.* 118:961–969.

Dionne, C.A., G. Crumley, F. Bellot, J.M. Kaplow, G. Searfoss, M. Ruta, W.H. Burgess, M. Jaye, and J. Schlessinger. 1990. Cloning and expression of two distinct high-affinity receptors cross reacting with acidic and basic fibroblast growth factors. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2685–2692.

Eisemann, A., J.A. Ahn, G. Graziani, S.R. Tronick, and D. Ron. 1991. Alternative splicing generates at least five different isoforms of the human basic-FGF receptor. *Oncogene.* 6:1195–1202.

Guimond, S., M. Maccarana, B.B. Olwin, U. Lindahl, and A.C. Rapraeger. 1993. Activating and inhibitory heparin sequences for FGF-2 (basic FGF). Distinct requirements for FGF-1, FGF-2, and FGF-4. *J. Biol. Chem.* 268: 23906–23914.

Habuchi, H., S. Suzuki, T. Saito, T. Tamura, T. Harada, K. Yoshida, and K. Kimata. 1992. Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. *Biochem. J.* 285:805–813.

Hochuli, E., H. Döbeli, and A. Schacher. 1987. New metal chelate adsorbant selective for proteins and peptide containing neighboring histidine residues. *J. Chromatogr.* 411:177–184.

Honma, Y., J. Okabe-Kado, M. Hozumi, Y. Uehara, and S. Mizumi. 1989. Induction of erythroid differentiation of K562 human leukemic cells by herbimycin A, an inhibitor of tyrosine kinase activity. *Cancer Res.* 49:331–334.

Ishihara, M., D.J. Tyrrell, G.B. Stauber, S. Brown, L.S. Cousens, and R.J. Stack. 1993. Preparation of affinity-fractionated, heparin-derived oligosaccharides and their effects on selected biological activities mediated by basic fibroblast growth factor. *J. Biol. Chem.* 268:4675–4683.

Johnson, D.E., J. Lu, H. Chen, S. Werner, and L.T. Williams. 1991. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanism for generating receptor forms that differ in their third immunoglobulin domain. *Mol. Cell. Biol.* 11:4627–4634.

Kan, M., F. Wang, J. Xu, J.W. Crabb, J. Hou, and W.L. McKeehan. 1993. An es-

sential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science (Wash. DC).* 259:1918–1921.

Kato, M., H. Wang, M. Bernfield, J.T. Gallagher, and J.E. Turnbull. 1994. Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J. Biol. Chem.* 269:18881–18890.

Keegan, K., D.E. Johnson, L.T. Williams, and M.J. Hayman. 1991. Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. *Proc. Natl. Acad. Sci. USA.* 88:1095–1099.

Kiefer, M.C., A. Baird, T. Nguyen, C. George-Nascimento, O.B. Mason, L.J. Boley, P. Valenzuela, and P.J. Barr. 1991. Molecular cloning of a human basic fibroblast growth factor receptor cDNA and expression of a biologically active extracellular domain in a baculovirus system. *Growth Factors.* 5:115–127.

Lories, V., J.-J. Cassiman, H. van den Berghe, and G. David. 1989. Multiple distinct membrane heparan sulfate proteoglycans in human lung fibroblasts. *J. Biol. Chem.* 264:7009–7016.

Lories, V., H. De Boeck, G. David, J.-J. Cassiman, and H. van den Berghe. 1987. Heparan sulfate proteoglycans of human lung fibroblasts. *J. Biol. Chem.* 262:854–859.

Maccarana, M., B. Casu, and U. Lindahl. 1993. Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* 268:23898–23905.

Mali, M., K. Elenius, H.M. Miettinen, and M. Jalkanen. 1993. Inhibition of basic fibroblast growth factor-induced growth promotion by overexpression of syndecan-1. *J. Biol. Chem.* 268:24215–24222.

Mali, M., P. Jaakkola, A.-M. Arvilommi, and M. Jalkanen. 1990. Sequence of human syndecan indicates a novel gene family of integral membrane proteoglycans. *J. Biol. Chem.* 265:6884–6889.

Marynen, P., J. Zhang, J.-J. Cassiman, H. van den Berghe, and G. David. 1989. Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. *J. Biol. Chem.* 264:7017–7024.

Mason, I.J. 1994. The ins and outs of fibroblast growth factors. *Cell.* 78:547–552.

Metz, C.N., G. Brunner, N.H. Choi-Muira, H. Nguyen, J. Gabrilove, I.W. Caras, N. Altszuler, D.B. Rifkin, E.L. Wilson, and M.A. Davitz. 1994. Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1741–1751.

Miki, T., D.P. Bottaro, T.P. Fleming, C.L. Smith, W.H. Burgess, A.M. Chan, and S.A. Aaronson. 1992. Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc. Natl. Acad. Sci. USA.* 89:246–250.

Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* 131:123–130.

Nugent, M.A., and E.R. Edelman. 1992. Kinetics of basic fibroblast growth factor to its receptor and heparan sulfate proteoglycan: a mechanism for cooperativity. *Biochemistry.* 31:8876–8883.

Nurcombe, V., M.D. Ford, J.A. Wildschut, and P.F. Bartlett. 1993. Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science (Wash. DC).* 260:103–106.

Ornitz, D.M., A. Yayon, J.G. Flanagan, C.M. Svahn, E. Levi, and P. Leder. 1992. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* 12:240–247.

Pantoliano, M.W., R.A. Horlick, B.A. Springer, D.E. Van Dyk, T. Tobery, D.R. Wetmore, J.D. Lear, A.T. Nahapetian, J.D. Bradley, and W.P. Sisk. 1994. Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry.* 33:10229–10248.

Papayannopoulou, T., B. Nakamoto, T. Yokochi, A. Chait, and R. Kannagi. 1983. Human erythroleukemia cell line (HEL) undergoes a macrophage-like shift with TPA. *Blood.* 62:832–845.

Partanen, J., T.P. Mäkelä, E. Eerola, J. Korhonen, H. Hirvonen, L. Claesson-Welsh, and K. Alitalo. 1991. FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1347–1354.

Rapraeger, A.C., A. Krufka, and B.B. Olwin. 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science (Wash. DC).* 252:1705–1708.

Richardson, J.M., A.O. Morla, and J.Y.J. Wang. 1987. Reduction in protein tyrosine phosphorylation during differentiation of human leukemia cell line K562. *Cancer Res.* 47:4066–4070.

Roghani, M., A. Mansukhani, P. Dell'Era, P. Bellosta, C. Basilico, D.B. Rifkin, and D. Moscatelli. 1994. Heparin increases the affinity of basic fibroblast growth factor for its receptor but is not required for binding. *J. Biol. Chem.* 269:3976–3984.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G. T. Horn, K.B. Mullis, and H.A. Ehrlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487–491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660–672.

Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

Seno, M., R. Sasada, T. Kurokawa, and K. Igarashi. 1990. Carboxyl-terminal structure of basic fibroblast growth factor significantly contributes to its affinity for heparin. *Eur. J. Biochem.* 188:239–245.

Shively, J.E., and H.E. Conrad. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. *Biochemistry.* 15:3932–3942.

Shworak, N.W., M. Shirakawa, S. Colliec-Jouault, J. Liu, R.C. Mulligan, L.K. Birinyi, and R.D. Rosenberg. 1994. Pathway-specific regulation of the synthesis of anticoagulantly active heparan sulfate. *J. Biol. Chem.* 269:24941–24952.

Spivak-Kroizman, T., M.A. Lemmon, I. Dikić, J.E. Ladbury, D. Pinchasi, J. Huang, M. Jaye, G. Crumley, J. Schlessinger, and I. Lax. 1994. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell.* 79:1015–1024.

Turnbull, J.E., D.G. Fernig, Y. Ke, M.C. Wilkinson, and J.T. Gallagher. 1992. Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* 267:10337–10341.

Tyrrell, D.J., M. Ishihara, N. Rao, A. Horne, M. C. Kiefer, G. B. Stauber, L.H. Lam, and R.J. Stack. 1993. Structure and biological activities of a heparin-derived hexasaccharide with high affinity for basic fibroblast growth factor. *J. Biol. Chem.* 268:4684–4689.

Walker, A., J.E. Turnbull, and J.T. Gallagher. 1994. Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor. *J. Biol. Chem.* 269:931–935.

Wennström, S., C. Sandström, and L. Claesson-Welsh. 1991. cDNA cloning and expression of a human FGF receptor which binds acidic and basic FGF. *Growth Factors.* 4:197–208.

Werner, S., D.S. Duan, C. de Vries, K.G. Peters, D.E. Johnson, and L. T. Williams. 1992. Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specificities. *Mol. Cell. Biol.* 12:82–88.

Yayon, A., M. Klagsbrun, J.D. Esko, P. Leder, and D.M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841–848.

Yayon, A., Y. Zimmer, G.H. Shen, A. Avivi, Y. Yarden, and D. Givol. 1992. A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold. *EMB (Eur. Mol. Biol. Organ.) J.* 11:1885–1890.

#12 attach.

Proteoglycans in the nervous system

Arthur D. Lander

Massachusetts Institute of Technology, Cambridge, USA

Proteoglycans are ubiquitous cell-surface and secreted glycoproteins that are involved in diverse cellular behaviors. The identities of several nervous system proteoglycans, including many of the major species in the mammalian brain, have recently come to light. In addition, recent studies have given new insights into the roles of proteoglycans in nervous system development and function.

Current Opinion in Neurobiology 1993, 3:716–723

Introduction

Proteoglycans (PGs) are found on the surfaces of all adherent cells, within intracellular vesicles, and in virtually all extracellular matrices (ECMs). They are evolutionarily ancient molecules, and play functional roles in the biology of growth factors, extracellular proteolysis, cell adhesion, lipoprotein metabolism, and virus entry into cells, as well as structural roles in maintaining the physical and mechanical properties of ECMs [1,2•,3,4].

Although many basic characteristics of PGs — their number, their structures, the exact nature of the functions they perform — are still slowly emerging, great progress has been made in recent years. With this recent burst of activity has come increasing recognition of the significance of PGs by neurobiologists, and increasing interest in the postulated roles PGs play in the nervous system. Some investigators have isolated monoclonal antibodies against nervous system molecules that have turned out to be PGs. Other investigators have become intrigued by the fact that many of the molecules that are thought to influence neuronal and glial cell behavior *in vivo*, especially during development, bind PGs. In the last few years, direct assaults on determining the structures of central nervous system (CNS) PGs have been undertaken by several groups. The purpose of this article is to review some of these recent results, and place them into the wider context of what PGs are, and how they are thought to function.

What are PGs?

A protein is called a PG if it contains a covalently attached glycosaminoglycan (GAG). GAGs are linear polysaccharides, typically 20–200 sugars in length, which are usually attached via a characteristic linkage region to serine residues. GAGs are built by the sequential addition of identical disaccharide units onto this linkage region. Only three types of disaccharide may be used, giving rise to three families of GAGs: the heparin/heparan family [D-glucuronic acid $\beta(1\rightarrow4)$ D-*N*-acetyl glucosamine $\alpha(1\rightarrow4)]_n$; the chondroitin/dermatan family [D-glucuronic acid $\beta(1\rightarrow3)$ D-*N*-acetyl galactosamine $\beta(1\rightarrow4)]_n$; and the keratan family [D-galactose $\beta(1\rightarrow4)$ D-*N*-acetyl glucosamine $\beta(1\rightarrow3)]_n$. The sugars of most GAGs are further chemically modified, typically in a sporadic fashion throughout the chain, by *O*-sulfation, *N*-deacetylation followed by *N*-sulfation, and/or epimerization (isomerization) of glucuronic acid to iduronic acid. Subsequently, GAGs are referred to as heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) or keratan sulfate (KS). The heparin/HS distinction and the CS/DS distinction only reflect differences in level of modification (i.e. heparin is more highly modified than most HS species; DS contains much more iduronate than CS). As each disaccharide in a GAG chain may be modified to a different degree, the large scale structures of GAGs can be exceedingly complex (e.g. in HS, which can be modified in up to five ways, a hexasaccharide can theoretically have over 30,000 possible chemical structures).

Products of several gene families, including secreted and membrane-inserted polypeptides, act as the core proteins of major PGs (Table 1). Some bear as few as one GAG chain, whereas others have over a hundred. Although the signals that specify whether a serine residue will bear a GAG are partially understood [2•], it is not known what controls the type of GAG synthesized: examples exist of cores that always bear one type of GAG, cores that bear different GAGs at different sites, and cores that bear different GAGs depending on the cell type in which they are expressed.

Abbreviations

CNS—central nervous system; CS—chondroitin sulfate; DS—dermatan sulfate; ECM—extracellular matrix; FGF—fibroblast growth factor; GAG—glycosaminoglycan; HS—heparan sulfate; KS—keratan sulfate; NCAM—neural cell adhesion molecule; NgCAM – neuron-glial cell adhesion molecule; PG —proteoglycan.

Table 1. Cloned PG core proteins.[a]

Cell-surface PGs	
	Syndecan family
	Syndecan (Syndecan-1)
	Fibroglycan (Syndecan-2)
	N-Syndecan Syndecan-3
	Ryudican Amphiglycan Syndecan-4
	Glypican family
	Glypican
	Cerebroglycan
	NG-2
	'Part-time PGs'[b]
ECM PGs	
	Aggrecan family
	Aggrecan
	Versican
	Neurocan
	Small, interstitial PG family
	Decorin
	Biglycan
	Fibromodulin
	Lumican
	Perlecan
	Type IX Collagen
Intravesicular PGs	
	Serglycin
	SV2

[a]Only shown are the obligate PG core proteins, i.e. those that invariably bear GAG chains. A small number of other cell-surface proteins bear GAG chains in some cells, but not others. [b]These 'part-time' PGs include CD44 and the type III transforming growth factor (TGF)-β receptor (reviewed in [1,2•,53]).

Cell surface PGs of the CNS

Early progress toward identifying cell surface PGs of the brain was made by Margolis' group, who detected a single major HSPG in adult brain membranes [5]. Later, Herndon and I [6] found evidence for CSPGs and other, less abundant, HSPGs in adult brain membranes, as well as additional major HSPGs that are present only during development. In the past year, the core proteins of several of these have been identified.

Glypican

Glypican was first identified as a surface HSPG core protein of human fibroblasts [7]. The mature polypeptide is ~53 kDa and is anchored in the plasma membrane by covalently attached glycosylphosphatidylinositol. Both the adult brain HSPG identified by Klinger *et al.* [5], and brain HSPG M12 identified by us [6], are the rat form of glypican ([8]; ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data). *In situ* hybridization studies in the adult brain and spinal cord indicate that glypican mRNA is expressed primarily, if not exclusively, by projection neurons in many, but not all parts of the CNS (ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data) (see Table 2). In the embryo, glypican is also strongly expressed in ventricular zones (regions undergoing neural precursor proliferation) throughout the neuraxis (Fig. 1).

Cerebroglycan

Cerebroglycan, previously called PG M13 [6], is an HSPG with a ~58 kDa core protein, and was first detected in the embryonic and newborn — but not adult — rat brain. Like glypican, it is glycosylphosphatidylinositol-anchored. In fact, glypican and cerebroglycan define a family of lipid-anchored HSPG cores, based on amino acid sequence similarity (CS Stipp, ED Litwack, AD Lander, unpublished data) (see Table 2). *In situ* hybridization studies indicate that cerebroglycan is transiently expressed by postmitotic neurons throughout the CNS (Fig. 1). Evidently, cerebroglycan mRNA appears in neurons shortly after terminal mitosis and disappears after neuronal migration and axon growth have been completed. Interestingly, cerebroglycan is not expressed outside the nervous system.

N-syndecan

N-syndecan (or syndecan-3) is one of four members of the syndecan family of transmembrane core proteins (Table 1). These polypeptides have short (~34 amino acids) cytoplasmic domains that are highly conserved among all family members, and overall sizes varying from 20 kDa (syndecans-2 and -4) to ≥ 42 kDa (syndecan-3). Their extracellular domains are poorly conserved among the different family members, or even for the same syndecan in different mammalian species. *N*-syndecan was cloned by Carey *et al.* [9••], who identified it in rat Schwann cell membranes (see Table 2). High levels of *N*-syndecan mRNA are also found in neonatal rat brain, as well as in many sites outside the nervous system. Expression of this molecule in rat brain peaks at birth, declining to undetectable levels thereafter. Early immunohistochemical studies suggest that this PG is associated with fiber tracts, but it is not yet known whether its source is neuronal or glial.

Syndecan-2

Syndecan-2, also known as fibroglycan, another member of the syndecan family, has not yet been isolated from the brain, but its mRNA has been found there (see Table 2). Based on electrophoretic behavior, syndecan-2 may correspond to brain PG M14 [6].

Table 2. PGs of the mammalian CNS.[a]

Name	Family	GAG	CNS Expression
Syndecan-3	Syndecan	HS	Transiently expressed in perinatal brain; widespread [9••]
Glypican[b]	Glypican	HS	Neuroepithelium; certain adult projection neurons [8][c]
Cerebroglycan[b]	Glypican	HS	Transiently expressed by newly post-mitotic neurons[d]
NG-2	NG-2	CS	O-2A progenitors [10]
Syndecan-2	Syndecan	HS	Unknown [2•]
Neurocan (1D1)	Aggrecan	CS	White matter of developing cerebellum; molecular layer of adult cerebellum. Mostly intracellular in adult [12••,18]
Versican	Aggrecan	CS	White matter [13•]
Aggrecan	Aggrecan	CS	Embryonic chick brain [14•]
Cat-301	Aggrecan?	CS	Subsets of neurons, cerebellum and spinal cord [15•,20]
PG-T1	?	CS	Widespread [16••,17•]
3H1	?	CS KS	Similar to neurocan [18]
3F8	?	CS	Concentrated in molecular layer of developing and adult cerebellum [18]
6B4	?	CS	Cerebellar and brainstem projection neurons [19•]
Unnamed	?	HS	Transient, in CNS fiber tracts [26••]
SV2 antigen	SV2	KS	Synaptic vesicles [27••]

[a]PGs are referred to by the names of their core proteins, and are grouped according to whether they are cell surface, extracellular matrix/soluble, or intravesicular molecules (see text). In many cases, information on CNS distribution has been based on the examination of only a few brain regions, and is therefore incomplete. [b]Data on distribution of glypican and cerebroglycan are based on *in situ* hybridization; most other data were obtained using antibodies. [c]ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data. [d]CS Stipp, ED Litwack, AD Lander, unpublished data.

NG2

NG2 is a transmembrane CSPG with a 300 kDa core protein [10]. In the brain it is associated with a population of glial precursor cells, the O-2A progenitors (see Table 2), that give rise to oligodendrocytes and a type of astrocyte. The very large core protein of NG2 suggests that it may serve functions other than just bearing CS chains. One such function appears to be the binding of type VI collagen [11].

ECM and 'soluble' PGs of the CNS

Many PGs can be extracted from the brain using physiological buffers without detergent; others require high salt or denaturing conditions. Although it has been argued that some of these molecules may reside in the cytoplasm of cells, most are probably loosely associated with the ECM.

Most of the PGs in these categories contain CS as their major GAG. Neurocan, a recently cloned CSPG, has a 136 kDa core protein, and contains ~3 CS chains [12••]. Its protein sequence places it in a family with aggrecan — the major ECM PG of cartilage — and versican, an ECM PG first found associated with fibroblasts. Like these other PGs, neurocan binds the ECM polysaccharide hyaluronic acid via a protein domain that is highly conserved in all three family members. Recent evidence suggests that versican and aggrecan are themselves expressed in the human and chicken brain, respectively [13•,14•]. The Cat-301 antigen is yet another large brain CSPG that binds hyaluronic acid, and immunological evidence suggests that it is related to aggrecan [15•]. One additional hyaluronic acid-binding CSPG, the T1 antigen, has been identified in brain, but at least the hyaluronic acid-binding region of this molecule is apparently unrelated to those of the aggrecan family [16••,17•]. Still other brain CSPGs have been identified with monoclonal antibodies, and remain to be fully characterized [14•,18,19•].

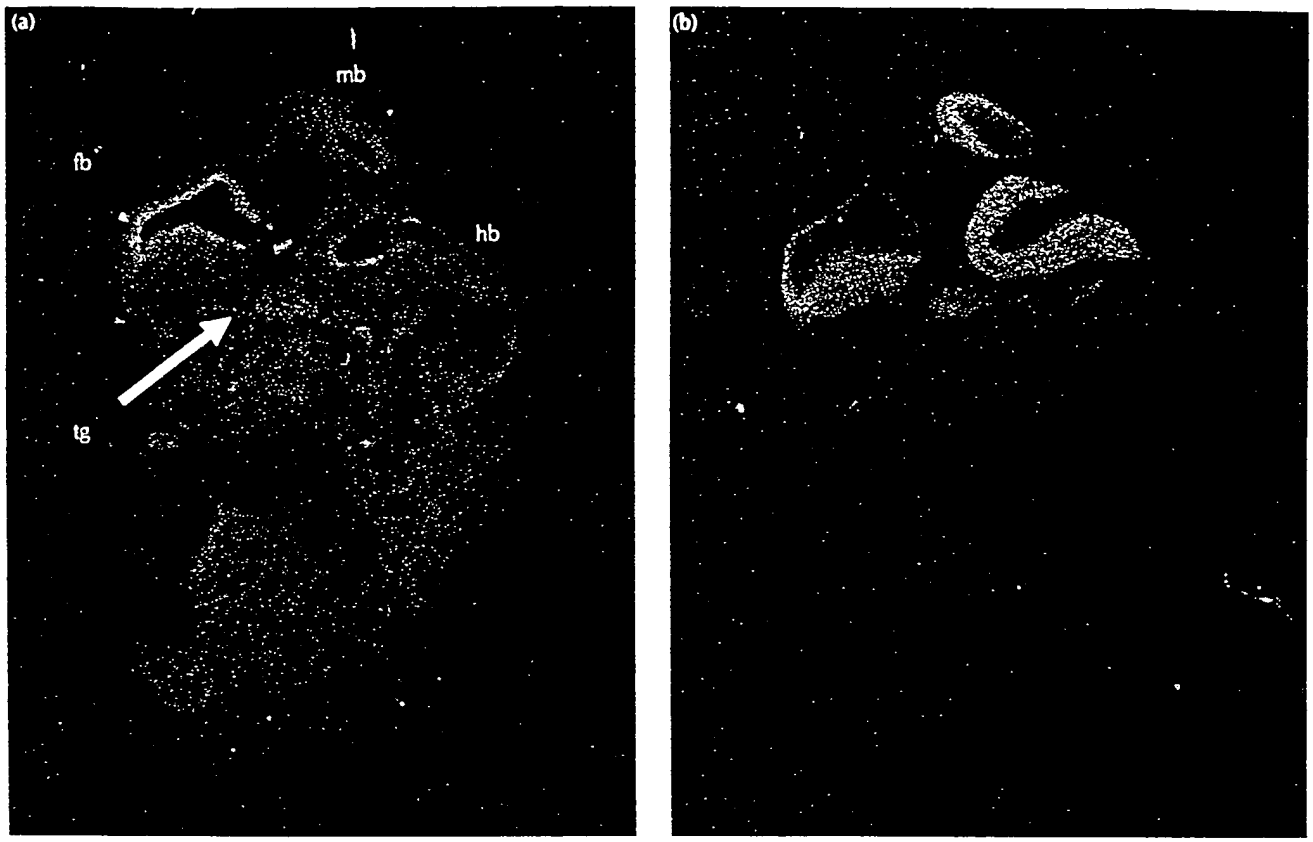


Fig. 1. Expression of glypican and cerebroglycan in the rat embryo. Adjacent sections of embryonic day 14 rats were hybridized with radiolabeled RNA probes specific for **(a)** glypican and **(b)** cerebroglycan mRNA. The images are reverse contrast prints of the resulting autoradiograms. Glypican expression is found throughout the embryo, but is particularly strong in the ventricular zones of the developing CNS. In contrast, cerebroglycan mRNA, which is found only in neural tissue, is not detected in ventricular zones but is found in the layers of immature neurons that form around those zones. In the adult brain, glypican is expressed by subpopulations of neurons, whereas cerebroglycan is absent. fb—forebrain; mb—midbrain; hb—hindbrain; tg—trigeminal ganglion.

The distributions of these CSPGs vary from remarkably uniform throughout the brain (PG T1) to remarkably cell type- and de opmental stage-specific. For example, Cat-301 appears around certain subsets of neurons only after activity-dependent critical periods in their development [20]. Another is transiently expressed during axon outgrowth by several types of neurons involved in the cerebellar mossy fiber system [19•].

As information on the distribution of these CSPG and CS KSPG core proteins accumulates, so has information on the distribution of different types of CS and KS chains. Several investigators have observed remarkable cell-type specificity in the binding of anti-CS and anti-KS monoclonal antibodies to brain sections (e.g. [21,22]). During cerebral cortex development, CS is found in the early proliferative neuroepithelium, then later in the marginal zone and subplate regions [23]. With the exception of the subplate, many of the locations of CS expression during development correlate with sites where axons do not grow. For example, CS, as well as KS, are strongly expressed in the roof plate of the spinal cord [24,25].

Recently, a report of an HSPG in brain ECM appeared [26••]. This molecule is found in basal laminae outside of and surrounding the chicken brain, but is also expressed transiently in many developing CNS axon tracts. The core protein size (250 kDa) and basal laminar distribution of this PG are reminiscent of perlecan, a major basement membrane PG, but perlecan itself is not found in S axon tracts.

Synaptic vesicle PGs

It has long been known that a PG is a major component of synaptic vesicles isolated from the electric organs of fishes. This PG was recently shown to be a transmembrane KSPG, and appears to be involved in acetylcholine transport into vesicles [27••]. Immunochemical data suggest that this molecule is present in many other types of synaptic vesicles, and might therefore play an important general role in transmitter uptake.

Roles of PGs in the nervous system

Insights into the functions of PGs in the nervous system have come by many routes, direct and indirect, and many of the conclusions are still somewhat preliminary. Highlights of what has been learned are summarized below.

The functions of a family of growth factors are dependent on PGs

All members of the fibroblast growth factor (FGF) family bind GAGs of the heparin/HS class, and apparently must do so to be biologically active [28,29]. Recent studies support a model in which cell-surface HSPGs bind both FGFs and FGF receptors simultaneously, facilitating their interaction [30]. It is known that at least three FGFs — FGF-1, -2 and -5 — are expressed in the nervous system and exert trophic effects on several classes of neurons [31–34,35••]. Recently, Nurcombe *et al.* [35••] have suggested that differences in the type of HS carried by a single core protein can render early neuroepithelial cells selectively responsive either to FGF-1 or to FGF-2. This proposition is supported by evidence in other systems that HS structure can impart specificity to HSPG function (e.g. [36,37•,38,39•]).

The kinetics of action of a family of protease inhibitors are dependent on PGs

The structurally related molecules antithrombin III, heparin cofactor II, and protease nexin I all bind and inactivate certain serine proteases (e.g. thrombin) much more rapidly when appropriate GAGs are present. To a large extent, GAGs act by simultaneously binding both protease and protease inhibitor, confining them to the same locality and thereby facilitating their interaction [38]. Of interest to neurobiologists, protease nexin I is abundantly expressed in the CNS, and is thought to regulate neurite outgrowth and neuronal migration [40].

Cell surface PGs participate in establishing cell–cell and cell–ECM contacts

Although cell surface PGs can apparently be the sole receptors for attachment to certain substrata [37•], PGs usually facilitate interactions mediated through other receptors, such as integrin-dependent cell attachment to ECM molecules [41], and neural cell adhesion molecule (NCAM)-dependent cell–cell adhesion [42,43]. A recent study suggests that cell surface HSPGs are especially important for the interaction of neural cells with fibronectin [44•]. As ECM and cell adhesion molecules are thought to provide important navigational cues to growing axons, the involvement of PGs with such molecules suggests a potential role for PGs in axon guidance. Recent studies in insects support this idea [45••].

ECM PGs regulate cell–cell and cell–matrix interactions

The core protein of at least one PG, perlecan, supports integrin-mediated cell attachment [46]. In contrast, several PGs inhibit the biological activities of ECM and cell adhesion molecules, at least *in vitro*. For example, adsorbed CSPGs or CS KSPGs can render culture substrata inhospitable for neurite growth [24,25]. Soluble CSPGs from rat brain also inhibit neurite outgrowth by PC12 cells [47]. Neurocan and the 3F8 CSPG of rat brain (but not aggrecan) inhibit homophilic NCAM and neuron-glial cell adhesion molecule (NgCAM)-binding [48•]. A HSPG released by Schwannoma cells specifically blocks the neurite outgrowth-promoting activity of laminin [49]. In some of these cases, the GAG chains of the PGs are required for these actions [24,25,49]; in others they are not [47,48•]. It is not yet known whether these phenomena are direct actions of PGs on neurons, or reflect effects of PGs on the physical characteristics of the culture substratum, so caution must be used in extrapolating these results to *in vivo* settings. Nonetheless, the distributions of some CSPGs are consistent with a 'barrier' function *in vivo* (see above). For example, in the developing retina a receding wave of CS expression marks a front of centripetally directed axons, suggesting that axons are guided by their avoidance of CS. Intriguingly, a CS-degrading enzyme disrupts the timing and direction of retinofugal axons in the developing rat retina [50••].

PGs are involved in the assembly of ECM, and act as binding sites for molecules that associate with the ECM

PGs bind virtually every major ECM component. In addition, molecules such as growth factors (e.g. FGFs) and enzymes (e.g. synaptic acetylcholinesterase) are often immobilized in ECMs through interactions with HSPGs [1,51]. The importance of PGs in ECM structure and function is illustrated by a muscle cell line that is defective in GAG biosynthesis [52•]. This cell line produces an abnormal basal lamina and, probably as a consequence, fails to form acetylcholine receptor clusters. The cells also fail to form such clusters in response to agrin, a GAG-binding ECM molecule that potently induces receptor clusters on normal muscle cells, and is thought to be involved in synaptogenesis *in vivo*.

Conclusions

Although much still needs to be learned about nervous system PGs, the identities of many of the major species in the brain are now known. Tracking down the functions of these molecules will probably not be easy. Their biological activities are likely to reside in their capacity to regulate, possibly in subtle ways, the functions of the molecules they bind. Moreover, the repertoire of molecules they bind will probably depend in part on the precise structures of their GAG chains, structures which defy easy analysis. Nevertheless, PGs are likely to continue to receive increasing attention in neurobiology, as their *in vivo* distributions and *in vitro* activities suggest that they are widely involved in nervous system development and function.

Acknowledgements

The author thanks ED Litwack and C Stipp for helpful comments and assistance in preparing the manuscript. This work was supported by NIH grant NS26862.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. RUOSLAHTI E: Proteoglycans in Cell Regulation. *J Biol Chem* 1989, 264:13369–13372.

2. • BERNFIELD M, KOKENYESI R, KATO M, HINKES MT, SPRING J, GALLO RL, LOSE EJ: Biology of the Syndecans: a Family of Transmembrane Heparan Sulfate Proteoglycans. *Annu Rev Cell Biol* 1992, 8:365–393.

A cogent review that brings together historical, structural and functional information about all members of the syndecan family, the major family of transmembrane HSPGs.

3. SHIEH MT, WUDUNN D, MONTGOMERY RI, ESKO JD, SPEAR PG: Cell Surface Receptors for Herpes Simplex Virus Are Heparan Sulfate Proteoglycans. *J Cell Biol* 1992, 5:1272–1281.

4. LANDER AD, CALOF AL: Extracellular Matrix in the Developing Nervous System. In *Molecular Genetics of Nervous System Tumors*. Edited by Levine AJ, Schmidek HH. New York: Wiley-Liss; 1993:341–355.

5. KLINGER MM, MARGOLIS RU, MARGOLIS RK: Isolation and Characterization of the Heparan Sulfate Proteoglycans of Brain. *J Biol Chem* 1985, 260:4082–4090.

6. HERNDON ME, LANDER AD: A Diverse Set of Developmentally Regulated Proteoglycans Is Expressed in the Rat Central Nervous System. *Neuron* 1990, 4:949–961.

7. DAVID G, LORIES V, DECOCK B, MARYNEN P, CASSIMAN J-J, VAN DEN BERGHE H: Molecular Cloning of a Phosphatidylinositol-Anchored Membrane Heparan Sulfate Proteoglycan From Human Lung Fibroblasts. *J Cell Biol* 1990, 111:3165–3176.

8. KARTHIKEYAN L, MAUREL P, RAUCH U, MARGOLIS RK, MARGOLIS RU: Cloning of a Major Heparan Sulfate Proteoglycan From Brain and Identification as the Rat Form of Glypican. *Biochem Biophys Res Commun* 1992, 188:395–401.

9. •• CAREY DJ, EVANS DM, STAHL RC, ASUNDI VK, CONNER KJ, GARBES P, CIZMECI-SMITH G: Molecular Cloning and Characterization of N-Syndecan, a Novel Transmembrane Heparan Sulfate Proteoglycan. *J Cell Biol* 1992, 117:191–201.

This paper presents the cloning of a partial *N*-syndecan (syndecan-3) cDNA, and identifies this molecule as a major HSPG of Schwann cells and developing brain.

10. NISHIYAMA A, DAHLIN KJ, PRINCE JT, JOHNSTONE SR, STALLCUP WB: The Primary Structure of NG2, a Novel Membrane-Spanning Proteoglycan. *J Cell Biol* 1991, 114:359–371.

11. STALLCUP WB, DAHLIN K, HEALY P: Interaction of the NG2 Chondroitin Sulfate Proteoglycan with Type VI Collagen. *J Cell Biol* 1990, 111:3177–3188.

12. •• RAUCH U, KARTHIKEYAN L, MAUREL P, MARGOLIS RU, MARGOLIS RK: Cloning and Primary Structure of Neurocan, a Developmentally Regulated, Aggretating Chondroitin Sulfate Proteoglycan of Brain. *J Biol Chem* 1992, 267:19536–19547.

The cloning of neurocan is described. The cDNA that was obtained encodes a protein similar to aggrecan and versican, including an amino-terminal hyaluronate binding domain, and carboxy-terminal epidermal growth factor (EGF)-like, lectin-like and complement regulatory-like domains. Evidence is presented that the adult brain contains only a truncated form of neurocan, lacking the amino-terminal region.

13. • PERIDES G, RAHEMTULLA F, LANE WS, ASHER RA, BIGNAMI A: Isolation of a Large Aggregating Proteoglycan from Human Brain. *J Biol Chem* 1992, 267:23883–23887.

Versican is isolated from human brain and shown to bind hyaluronic acid. The relationship of versican to GHAP (glial hyaluronic acid binding protein) is also discussed. GHAP, a previously characterized protein, appears to be an amino terminal fragment of versican.

14. • KRUEGER R, HENNIG AK, SCHWARTZ NB: Two Immunologically and Developmentally Distinct Chondroitin Sulfate Proteoglycans in Embryonic Chick Brain. *J Biol Chem* 1992, 267:12149–12161.

One of only a few studies to look at PGs in the avian brain. Aggrecan and its mRNA are found to be transiently expressed in the embryonic chick brain. Unlike cartilage aggrecan, the brain form contains only CS and not KS. Also detected in chick brain was a PG with a similar core size, but that can be distinguished from aggrecan by the presence of both CS and KS chains, reactivity with the HNK-1 monoclonal antibody, and lack of developmental regulation.

15. • FRYER HJL, KELLY GM, MOLINARO L, HOCKFIELD, S: The High Molecular Weight Cat-301 Chondroitin Sulfate Proteoglycan from Brain is Related to the Large Aggregating Proteoglycan from Cartilage, Aggrecan. *J Biol Chem* 1992, 267:9874–9883.

The Cat-301 immunoreactive CSPG of brain is shown to have properties similar to those of aggrecan. Also, authentic aggrecan from cartilage is shown to react with Cat-301, as well as to another monoclonal antibody that recognizes the same brain PG as Cat-301. Nevertheless, the Cat-301 antigen can be distinguished from aggrecan by virtue of containing few, if any, KS chains and having a lower buoyant density (indicative of a lower carbohydrate protein ratio) than aggrecan. The data suggest that Cat-301 is either a modified form of aggrecan, or a new member of the aggrecan family.

16. •• IWATA M, CARLSON S: A Large Chondroitin Sulfate Proteoglycan Has the Characteristics of a General Extracellular Matrix Component of Adult Brain. *J Neurosci* 1993, 13:195–207.

The T1 antigen is a large CSPG that can only be extracted from rat brain with chaotropic agents (e.g. guanidine). It appears to be expressed in all parts of the CNS, and is therefore proposed to be a general ECM component. It fails to react with an antiserum directed against a 15 amino acid sequence that is present in the hyaluronic acid-binding domain of aggrecan, is 100% conserved in versican, and 80% conserved in neurocan. This suggests that the T1 antigen may not belong to the aggrecan family.

17. • IWATA M, WIGHT TN, CARLSON SS: A Brain Extracellular Matrix Proteoglycan Forms Aggregates with Hyaluronan. *J Biol Chem* 1993, 268:15061–15069.

Even though the T1 antigen is thought to be unrelated to aggrecan and veriscan, it too binds hyaluronic acid. Using affinity co-electrophoresis, a ~1 nM dissociation constant for hyaluronic acid was measured.

18. RAUCH U, GEO P, JANETZKO A, FLACCUA A, HILGENBERG L, TEKOTTE H, MARGOLIS RK, MARGOLIS RU: Isolation and Characterization of Developmentally Regulated Chondroitin Sulfate and Chondroitin/Keratan Sulfate Proteoglycans of Brain Identified with Monoclonal Antibodies. *J Biol Chem* 1991, 266:14785–14801.

19. • MAEDA N, MATSUI F, OOHIRA A: A Chondroitin Sulfate Proteoglycan That Is Developmentally Regulated in the Cerebellar Mossy Fiber System. *Dev Biol* 1992, 151:564–574.

Monoclonal antibodies were prepared using soluble brain PGs as immunogen. An antibody (6B4) is described that recognizes a CSPG with a 250 kDa core protein. Using immunohistochemistry, the antigen was detected around cerebellar Purkinje cells, Golgi cells and deep cerebellar neurons, as well as in brainstem nuclei that project mossy fibers to the cerebellum. Transient immunoreactivity was also found on ponto-cerebellar fibers during development.

20. ZAREMBA S, NAEGELE J, BARNSTABLE C, HOCKFIELD S: Neuronal Subsets Express Multiple High-Molecular-Weight Cell-Surface Glyconjugates Defined by Monoclonal Antibodies Cat-301 and VC1.1. *J Neurosci* 1990, 10:2985–2995.

21. FUJITA SC, TADA Y, MURAKAMI F, HAYASHI M, MATSUMURA M: Glycosaminoglycan-Related Epitopes Surrounding Different Subsets of Mammalian Central Neurons. *Neurosci Res* 1989, 7:117–130.

22. BERTOLOTTO A, ROLCA G, CANAVESE G, MIGHELI A, SCHIFFER D: Chondroitin Sulfate Proteoglycan Surrounds a Subset of Human and Rat CNS Neurons. *J Neurosci Res* 1991, 29:225–234.

23 SHEPPARD AM, HAMILTON SK, PEARLMAN AL: Changes in the Distribution of Extracellular Matrix Components Accompany Early Morphogenetic Events of Mammalian Cortical Development. *J Neurosci* 1991, 11:3928–3942.

24. SNOW DM, LEMMON V, CARRINO DA, CAPLAN AI, SILVER J: Sulfated Proteoglycans in Astroglial Barriers Inhibit Neurite Outgrowth *in Vitro*. *Exp Neurol* 1990, 109:111–130.

25. SNOW DM, WATANABE M, LETOURNEAU PC, SILVER J: A Chondroitin Sulfate Proteoglycan May Influence the Direction of Retinal Ganglion Cell Outgrowth. *Development* 1991, 113:1473–1485.

26. •• HALFTER W: A Heparan Sulfate Proteoglycan in Developing Avian Axonal Tracts. *J Neurosci* 1993, 13:2863–2873.
A search for monoclonal antibodies against retinal basal lamina and optic nerve turns up four antibodies that recognize a single, large HSPG. Although this molecule exhibits biochemical properties similar to those of perlecan, its expression in CNS fiber tracts indicates that is not identical to perlecan. Until this report, it was not clear that any HSPGs were present in the ECM of the brain. Interestingly, like several other ECM molecules (e.g. laminin and fibronectin), expression of this PG in the parenchyma of the brain appears to be transient.

27. •• SCRANTON TW, IWATA M, CARLSON SS: The SV2 Protein of Synaptic Vesicles Is a Keratan Sulfate Proteoglycan. *J Neurochem* 1993, 61:29–44.
An important clarification of a complicated story. Two synaptic vesicle molecules, a large PG and a major vesicle glycoprotein called SV2 are shown to be forms of the same integral membrane protein, bearing either long or short KS chains. This helps to bring together findings in other labs that the synaptic vesicle PG is involved in acetylcholine transport, and that the SV2 protein is evolutionarily related to bacterial transport proteins.

28. YAYON A, KLAGSBRUN M, ESKO JD, LEDER P, ORNITZ, DM: Cell Surface, Heparin-like Molecules are Required for Binding of Basic Fibroblast Growth Factor to its High Affinity Receptor. *Cell* 1991, 64:841–848.

29. RAPRAEGER AC, KRUFKA A, OLWIN BB: Requirement of Heparan Sulfate for bFGF-Mediated Fibroblast Growth and Myoblast Differentiation. *Science* 1991, 252:1705–1708.

30. KAN M, WANG F, XU J, CRABB JW, HOU J, MCKEEHAN WL: An Essential Heparin-Binding Domain in the Fibroblast Growth Factor Receptor Kinase. *Science* 1993, 259:1918–1921.

31. WALICKE P: Basic and Acidic Fibroblast Growth Factors Have Trophic Effects on Neurons from Multiple CNS Regions. *J Neurosci* 1988, 8:2618–2627.

32. ELDE R, CAO Y, CINTRA A, BRELJE TC, PELTO-HUIKKO M, JUNTTILA T, FUXE K, PETTERSSON RF, HOKFELT T: Prominent Expression of Acidic Fibroblast Growth Factor in Motor and Sensory Neurons. *Neuron* 1991, 7:349–364.

33. GÓMEZ-PINILLA F, LEE JW-K, COTMAN CW: Basic FGF in Adult Rat Brain: Cellular Distribution and Response to Entorhinal Lesion and Fimbria-Fornix Transection. *J Neurosci* 1992, 12:345–355.

34. HUGHES RA, SENDTNER M, GOLDFARB M, LINDHOLM D, THOENEN H: Evidence that Fibroblast Growth Factor 5 is a Major Muscle-Derived Survival Factor for Cultured Spinal Motoneurons. *Neuron* 1993, 10:369–377.

35. •• NURCOMBE V, FORD MD, WILDSCHUT JA, BARLETT PF: Developmental Regulation of Neural Response to FGF-1 and FGF-2 by Heparan Sulfate Proteoglycan. *Science* 1993, 260:103–106.
In this provocative study, an HSPG was isolated from mouse neuroepithelial cells at two stages of development, and tested for binding to FGF-1 and FGF-2. HSPG from embryonic day 9 cells preferentially bound FGF-2, whereas HSPG from day 11 cells preferentially bound FGF-1. Similar specificity was seen in the ability of the HSPG samples to potentiate the actions of FGF-1 and FGF-2. The developmental switch in binding coincides with a switch in expression of FGFs in the neuroepithelium, from FGF-2 to FGF-1, over the same period.

36. SALMIVIRTA M, ELENIUS K, VAINIO S, HOFER U, CHIQUET EHRISMANN R, THESLEFF I, JALKANEN M: Syndecan from Embryonic Tooth Mesenchyme Binds Tenascin. *J Biol Chem* 1991, 266:7733–7739.

37. • SANDERSON RD, SNEED TB, YOUNG LA, SULLIVAN GL, LANDER AD: Adhesion of B Lymphoid (MPC-11) Cells to Type I Collagen is Mediated by the Integral Membrane Proteoglycan, Syndecan. *J Immunol* 1992, 148:3902–3911.
This study demonstrates that a cell surface HSPG can directly mediate cell adhesion, and that a single HSPG can have different affinities for an ECM ligand, depending, presumably, on the structure of its GAG chains.

38. • OLSON ST, BJORK I: Role of Protein Conformational Changes, Surface Approximation and Protein Cofactors in Heparin-Accelerated Antithrombin-Proteinase Reactions. In *Heparin and Related Polysaccharides*. Edited by Lane DA. New York: Plenum Press; 1992:155–165.

39. • SAN ANTONIO JD, SLOVER J, LAWLER J, KARNOVSKY MJ, LANDER AD: Specificity in the Interactions of Extracellular Matrix Proteins with Subpopulations of the Glycosaminoglycan Heparin. *Biochemistry* 1993, 32:4746–4755.
A direct demonstration that some ECM molecules (e.g. laminin, fibronectin and type I collagen) can bind preferentially to subpopulations of the GAG heparin. The implication is that features in the structure of heparin HS determine binding specificity. Thus, carbohydrate structure is likely to regulate the functions of HSPGs.

40. LINDNER J, GUENTHER J, NICK H, ZINSER G, ANTONICEK H, STAECHNER M, MONARD D: Modulation of Granule Cell Migration by a Glia-Derived Protein. *Proc Natl Acad Sci USA* 1986, 83:4568–4571.

41. WOODS A, COUCHMAN JR: Heparan Sulphate Proteoglycans and Signalling in Cell Adhesion. In *Heparin and Related Polysaccharides*. Edited by Lane DA. New York: Plenum Press; 1992:87–96.

42. COLE GJ, LOEWY A, GLASER L: Neuronal Cell-Cell Adhesion Depends on Interactions of N-CAM with Heparin-Like Molecules. *Nature* 1986, 320:445–447.

43. REYES AA, AKESON R, BREZINA L, COLE GJ: Structural Requirements for Neural Cell Adhesion Molecule-Heparin Interaction. *Cell Regul* 1990, 1:567–576.

44. • HAUGEN PK, LETOURNEAU PC, DRAKE SL, FURCHT LT, MCCARTHY JB: A Cell-Surface Heparan Sulfate Proteoglycan Mediates Neural Cell Adhesion and Spreading on a Defined Sequence from the C-Terminal Cell and Heparin Binding Domain of Fibronectin, FN-C/H II. *J Neurosci* 1992, 12:2597–2608.
Enzymatic removal of HS from rat neuroblastoma cells inhibited their attachment and spreading on fibronectin fragments and peptides, as did antibodies directed against a mouse melanoma HSPG.

45. •• WANG L, DENBURG J: A Role for Proteoglycans in the Guidance of a Subset of Pioneer Axons in Cultured Embryos of the Cockroach. *Neuron* 1992, 8:701–714.
Axons in the developing insect nervous system follow highly stereotyped pathways. Using a semi-*in vitro* system, evidence was found that heparin and HS specifically perturb certain axon guidance decisions. Similar perturbations were produced by two HS-degrading enzymes.

46. HAYASHI K, MADRI JA, YURCHENCO PD: Endothelial Cells Interact with the Core Protein of Basement Membrane Perlecan through β1 and β3 Integrins: an Adhesion Modulated by Glycosaminoglycan. *J Cell Biol* 1992, 119:945–959.

47. OOHIRA A, MATSUI F, KATOH-SEMBA R: Inhibitory Effects of Brain Chondroitin Sulfate Proteoglycans on Neurite Outgrowth from PC12D Cells. *J Neurosci* 1991, 11:822–827.

48. • GRUMET M, FLAccus A, MARGOLIS RU: Functional Characterization of Chondroitin Sulfate Proteoglycans of Brain: Interactions with Neurons and Neural Cell Adhesion Molecules. *J Cell Biol* 1993, 120:815–824.
Evidence is presented that the brain CSPGs neurocan (1D1) and 3F8 inhibit homophilic NCAM–NCAM and NgCAM–NgCAM binding (whereas aggrecan does so poorly), and that neurons exhibit some ability to attach to the core proteins of these PGs.

49. MUIR D, ENGVALL E, VARON S, MANTHORPE M: Schwannoma Cell-Derived Inhibitor of the Neurite-Promoting Activity of Laminin. *J Cell Biol* 1989, 109:2353–2362.

50. •• BRITTIS PA, CANNING DR, SILVER J: Chondroitin Sulfate as a Regulator of Neural Patterning in the Retina. *Science* 1992, 255:733–736.

This paper raises the provocative hypothesis that the disappearance of CS from the developing mammalian retina sets up a spatial gradient that guides ganglion cell axons toward the optic nerve head. Previous *in vitro* studies had suggested that CS repels retinal axons. In this study, evidence is presented that newly differentiating ganglion cells find themselves within a steep CS gradient oriented away from the optic disk. Evidence is also presented that exposure of developing retinas to a CS-degrading enzyme causes large perturbations in ganglion cell axon guidance, as well as causing ectopic differentiation of ganglion cells.

51. BRANDAN E, MALDONADO M, GARRIDO J, INESTROSA N: Anchorage of Collagen-Tailed Acetylcholinesterase to the Extracellular Matrix Is Mediated by Heparan Sulfate Proteoglycans. *J Cell Biol* 1985, 101:985–992.

52. • GORDON H, LUPA M, BOWEN D, HALL Z: A Muscle Cell Variant Defective in Glycosaminoglycan Biosynthesis Forms Nerve-Induced but Not Spontaneous Clusters of the Acetylcholine Receptor and the 43 kDa Protein. *J Neurosci* 1993, 13:586–595.

Acetylcholine receptor clustering by muscle cells is an important model for early steps in synaptogenesis. In this paper, S27, a variant of C2 mouse myoblasts that is deficient in GAG biosynthesis, is described. Unlike the parent line, S27 cells fail to form spontaneous or agrin-induced acetylcholine receptor clusters. In addition, basal lamina components (e.g. laminin) are also reduced on the surface of these cells. Interestingly, S27 cells are capable of forming acetylcholine receptor clusters at sites of contact by neuronal processes. Possible explanations for the different responses of these cells to clustering signals are discussed.

53. GALLAGHER JT: The Extended Family of Proteoglycans: Social Residents of the Pericellular Zone. *Curr Opin Cell Biol* 1989, 1:1201–1218.

AD Lander, Department of Brain and Cognitive Sciences, E25-435, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

Heparan sulfate: a piece of information

MARKKU SALMIVIRTA, KERSTIN LIDHOLT, AND ULF LINDAHL[1]

Department of Medical and Physiological Chemistry, Uppsala University, S-75123 Uppsala, Sweden

ABSTRACT **The sulfated glycosaminoglycans, heparan sulfate and heparin, are increasingly implicated in cell-biological processes such as cytokine action, cell adhesion, and regulation of enzymic catalysis. These activities generally depend on interactions of the polysaccharides with proteins, mediated by distinct saccharide sequences, and expressed at various levels of specificity, selectivity, and molecular organization. The formation of heparin/heparan sulfate in the cell requires an elaborate biosynthetic machinery, that is conceived in terms of a novel model of glycosaminoglycan assembly and processive modification. Recent advances in the identification and molecular analysis of the enzymes and other proteins involved in the biosynthesis provide novel tools to study the regulation of the process, presently poorly understood, at the subcellular and cellular levels. The potential medical importance of heparin-related compounds is likely to promote the biotechnological exploitation of components of the biosynthetic machinery.—Salmivirta, M., Lidholt, K., Lindahl, U. Heparan sulfate: a piece of information. *FASEB J.* 10, 1270–1279 (1996)**

Key Words: heparin · GAG · proteoglycan · enzyme · polysaccharide-protein interactions

HEPARIN, A MAMMALIAN glycosaminoglycan (GAG),[2] has the highest negative charge density of any known biological macromolecule. It thus is prone to ionic interaction with a variety of proteins such as enzymes, enzyme inhibitors, extracellular-matrix proteins, various cytokines, and others (1). Such interaction is exploited in the purification of "heparin-binding proteins," which are adsorbed to immobilized heparin at low ionic strength and subsequently eluted with salt. The appreciable purification often achieved suggests an element of selectivity beyond that expected for simple cation-exchange chromatography.

Heparin is isolated on a commercial basis from animal tissues (pig intestinal mucosa; bovine lung) and is used in the clinic as an antithrombotic drug. In the intact tissue it is confined to mast cells, where it is stored in cytoplasmic granules. Heparan sulfate (HS), on the other hand, has ubiquitous distribution on cell surfaces and in the extracellular matrix. It is generally less sulfated than heparin and has a more varied structure. Interactions between HS and specified proteins are being increasingly implicated in a variety of physiological processes, such as cell adhesion, enzyme regulation, cytokine action, etc. (1).

Heparin and HS are both synthesized as proteoglycans (PGs), which consist of GAG chains covalently bound to a protein core. A single protein, serglycin, has been identified as the protein constituent of heparin PGs, whereas a variety of proteins provide core structures of HS PGs (2–4). Biosynthesis of either heparin or HS PGs involves the formation of an initial, simple GAG structure, composed of alternating D-glucuronic (GlcA) and N-acetyl-D-glucosamine (GlcNAc) units, joined by 1 → 4 linkages (Fig. 1*A*). This structure may then be modified through a series of reactions that ultimately result in the formation of -IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)- sequences [where L-iduronic acid (IdoA) is the C5-epimerization product of GlcA] (Fig. 1*B*). This process, which generates the most abundant disaccharide unit in heparin, will be referred as heparin "default modification."

Heparin, and in particular, HS, contain structures that deviate from the product of default modification. Such structures arise through "modulated modification," which may differ from the default process in either of two ways. The pathway may be incomplete, due to lack of all (Fig. 1*A*) or some of the reactions, or it may involve additional reactions. Some structures generated through modulated polymer modification are shown in Fig. 1*C–F*.

POLYSACCHARIDE-PROTEIN INTERACTIONS

Binding of heparin/HS sequences to proteins is generally (although not exclusively) ionic, and thus involves positively charged, usually clustered, amino acid residues in the protein components. Attempts to define polypeptide consensus sequences for heparin binding have yielded partly contradictory results (5). Conversely, the anionic

[1]To whom correspondence and reprint requests should be addressed, at: Department of Medical and Physiological Chemistry, Uppsala University, Biomedical Center, P.O. Box 575, S-75123 Uppsala, Sweden.

[2]Abbreviations: FGF, fibroblast growth factor; GAG, glycosaminoglycan; GalNAc, 2-acetamido-2-deoxy-D-galactose (N-acetylgalactosamine); GlcNAc, 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine); $GlcNH_3^+$, N-unsubstituted glucosamine; GlcA, D-glucuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PG, proteoglycan.

0892-6638/96/0010-1270/$01.50

protein binding domains of polysaccharide chains may differ with regard to structure, degree of binding specificity, and organization at the macromolecular level (**Fig. 2**). Simple interactions involve single binding sites on the GAG chain (Fig. 2*A*) and on the protein moiety. Ternary complexes may contain two (identical or distinct) proteins bound to separate domains on the same GAG chain (Fig. 2*B*). Finally, different protein binding domains may be located on separate GAG chains that are bound to a common core protein in a PG (Fig. 2*C*).

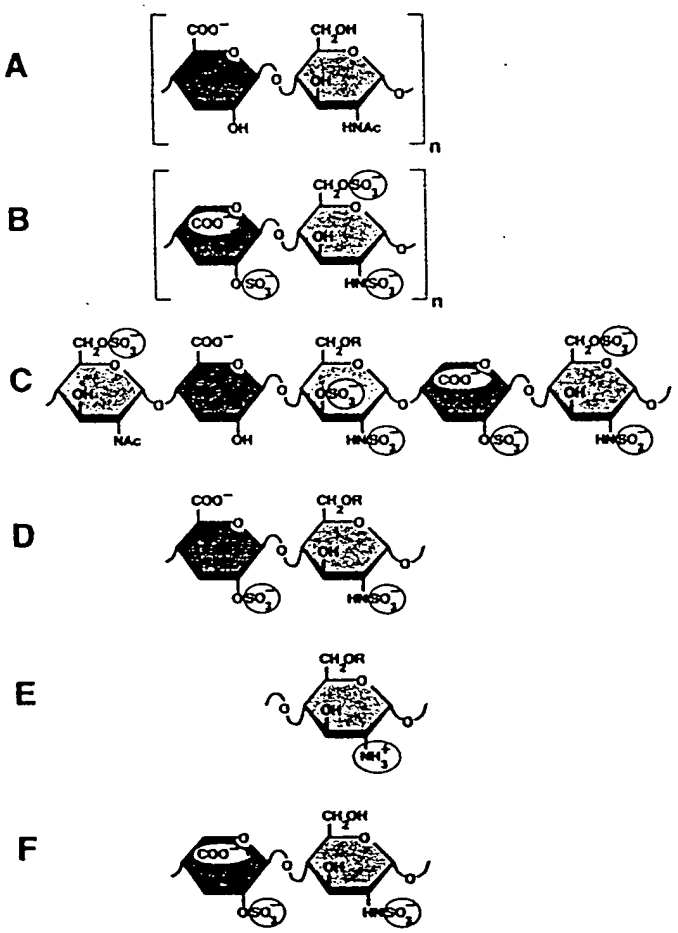


Figure 1. Examples of heparin/HS structures produced by "default" and "modulated" polymer modification. The initial polymerization product is a repeat of alternating GlcA and GlcNAc units (*A*). Approximately 80% of such disaccharides in heparin, but generally < 10% in HS, undergo default modification involving N-deacetylation and N-sulfation of GlcN-units, C-5 epimerization of GlcA to IdoA, and O-sulfation at two positions, yielding the -IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)- disaccharide unit (*B*). Products of modulated modification include the "unique" 3-O-sulfated GlcNSO_3 unit in the middle of the antithrombin binding pentasaccharide sequence (*C*). The -GlcA(2-OSO_3)-GlcNSO_3- disaccharide unit (*D*) is present in different HS species in highly variable amounts. A small proportion of GlcN residues in heparin and HS contain unsubstituted amino groups (*E*). The -IdoA(2-OSO_3)-GlcNSO_3- unit (*F*) is a common product of modulated (restricted) modification typical for HS. Hexuronic acid residues are shown in blue, glucosamine units are green, and sulfate groups yellow. For further information, see the text.

Protein binding regions generated by default modification

Interactions between heparin/HS and proteins generally depend on the presence of sulfate groups. Although this requirement is readily demonstrated by using chemically desulfated GAG preparations, it is more difficult to pinpoint those sulfate groups that are actually essential for binding. Saccharide sequences composed of repeating, default-modified disaccharide units, -[IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)]$_n$-, abundant in heparin and frequently found within the N-sulfated block regions of HS chains (6), bind to many proteins. Still, the precise requirement for individual sulfate groups within these sequences may vary from one "heparin binding" protein to another.

The enzyme lipoprotein lipase binds to HS chains of PGs at the surface of vascular endothelial cells, with marked preference for a saccharide sequence consisting exclusively of the trisulfated disaccharide unit (7). Other proteins, such as thrombin (8) and platelet factor 4 (9), bind to the same sequence in seemingly nonspecific fashion. The problem of specificity was highlighted in a series of studies involving members of the fibroblast growth factor (FGF) family. These proteins all bind heparin, apparently via the same sequence of default-modified (trisulfated) disaccharide units. However, attempts to define the minimal binding sequence for FGF-2 (basic FGF) revealed a pentasaccharide structure in which the essential sulfate groups were limited to a single IdoA 2-O-sulfate and one or two N-sulfate groups (10, 11). The remaining [IdoA(2-OSO_3) and GlcN(6-OSO_3)] O-sulfate groups of the fully sulfated heparin structure would seem not to contribute to or interfere with FGF-2 binding.

Protein binding regions generated by modulated modification

A concept of protein binding to the default-modified heparin sequence, but with selective involvement of sulfate groups, has intriguing implications. Indeed, the available data suggest that binding of different members of the FGF family may require different combinations of sulfate groups, hence different saccharide sequences (5, 12). Such sequences may well all be represented, albeit in "hidden" form, by the same fully sulfated, default-modified heparin structure shown in Fig. 1*B*, given the proviso that sulfate groups other than those of the implicated minimal sequences will not interfere with protein binding. Conceivably, however, they may also be differentially expressed in separate GAG chains of the HS type (**Fig. 3**). The generation of such specific saccharide ligands would require selective restriction of polymer modification. Consequently, minimal sequences are more likely to occur in HS than in heparin due to the more variable, and generally lower, degree of modification of the former species.

Modulated polymer modification may also result in the formation of rare ("unique") structural components that

are introduced through distinct reactions. An example of such a marker component that is implicated with a defined biological function is the 3-O-sulfated GlcN unit, which is located in the antithrombin binding pentasaccharide sequence of heparin and HS (Fig. 1*C*) and is essential to the blood anticoagulant activity of the polysaccharides (reviewed in ref 8). Another generally minor and variable component is the 2-O-sulfated GlcA unit (Fig. 1*D*), which contributes to the structural distinction between HS species from different sources. A -GlcA(2-OSO_3)-GlcNSO_3- disaccharide unit thus accounts for as much as 11% of the total N-sulfated disaccharide units of HS from adult human cerebral cortex, but is virtually absent in the corresponding neonatal material as well as in HS preparations from other adult tissues, such as the arterial wall (13). These findings suggest an organ-specific and age-related control of GlcA 2-O-sulfation. Even though the precise functional role of the sulfated GlcA residue is unknown, previous studies of cultured hepatocytes revealed a nuclear pool of HS with a strikingly high content of the -GlcA(2-OSO_3)-GlcNSO_3(6-OSO_3)- disaccharide unit (14). Nuclear HS was tentatively implicated with the control of cell proliferation. Heparin [which also contains small amounts of GlcA(2-OSO_3) units (2)] is known to bind the transcription factors Fos and Jun and to inhibit their effects on gene expression (15).

A small proportion of GlcN residues in heparin and HS preparations have unsubstituted amino groups (Fig. 1*E*). Recent immunohistochemical work established that the N-unsubstituted GlcN ($GlcNH_3^+$) units occur in native HS and are not due to preparation artifacts (as was tacitly assumed in the past) (16). Moreover, an antibody recognizing $GlcNH_3^+$-containing epitopes stained HS in glomerular basement membranes, but not that in tubular basement membranes of the rat kidney, suggesting a selective expression of the epitope. A possible clue to the functional role of this structure emerged through the finding that L-selectin preferentially bound to $GlcNH_3^+$-containing HS PGs (17). However, chemical N-acetylation of such PG did not impede L-selectin binding. It was proposed that the presence of a $GlcNH_3^+$ unit might regulate the biosynthetic modification of surrounding saccharide sequences, thus creating a structure recognized by L-selectin.

Multiple interaction sites

HS modulates the biological activity of interferon-γ by interacting with the dimeric cytokine. Interferon binding HS fragments, encompassing as many as 40–50 monosaccharide units, were shown to consist of two terminal sulfated domains, each binding to one interferon-γ monomer, separated by a nonsulfated GlcA-rich sequence (18). This finding points to an important general concept: properly spaced sequences of the appropriate structure along a GAG chain may form functional domains that act in a concerted manner (Fig. 2*B*). Such domains may bind to identical peptide sites, as in the case of interferon-γ, or to different sites, as in the heparin-antithrombin-thrombin interaction (8). Whereas antithrombin binds to a specific pentasaccharide sequence (see above), a much longer saccharide (minimal size ~18 monosaccharide units) is required to induce thrombin inhibition. This observation reflects the requirement for a ternary complex in which not only antithrombin but also thrombin bind to the polysaccharide chain (19).

An analogous mode of interactions has been proposed for the binding of heparin/HS to FGF-2 and its cell-surface tyrosine kinase type receptor (20). The minimal heparin/HS sequence required to promote FGF-2-induced cell proliferation consists of ~12 monosaccharide units, more than twice the size of the pentasaccharide region that actually binds the growth factor (see above). One possible explanation to this finding is that binding of two growth factor molecules to adjacent sites on the GAG chain will promote receptor dimerization, as required for receptor activation (21). However, there is evidence for

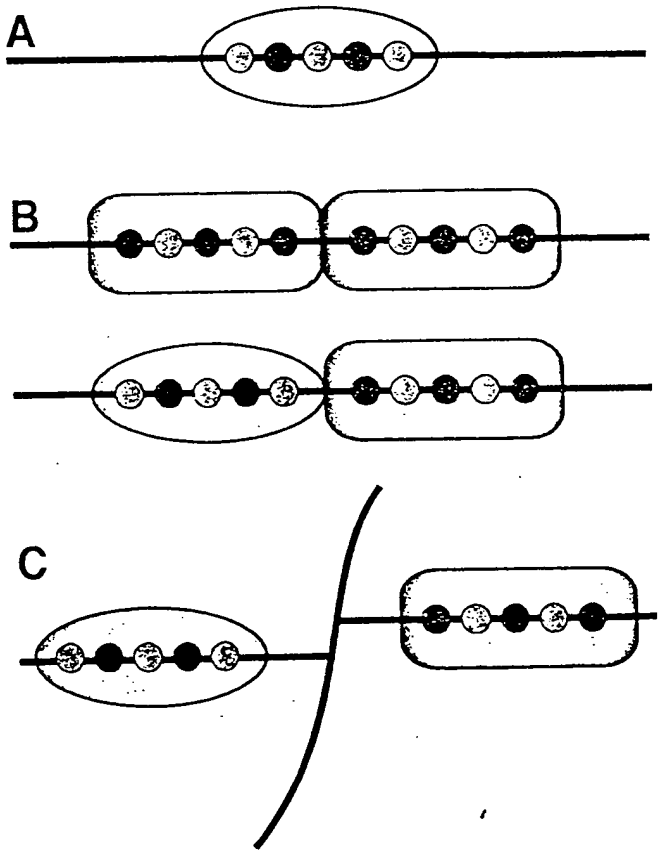


Figure 2. Models of differentially organized single and multiple protein binding domains in heparin/HS. Single protein binding sites along a polysaccharide chain (*A*) may vary in size and composition depending on ligand specificity. Two similar sequences located adjacent to each other (upper model in panel *B*) may form a composite binding domain for two identical ligands, such as the subunits of a protein dimer. An analogous arrangement of two nonidentical domains will promote the formation of ternary complexes between the saccharide chain and two distinct proteins (lower model in panel *B*). Finally, similar or distinct, single or multiple binding domains may reside in separate HS chains of a PG (*C*), thus providing a functional versatility for a PG molecule that is not possessed by its individual HS chains. For further information, see the text.

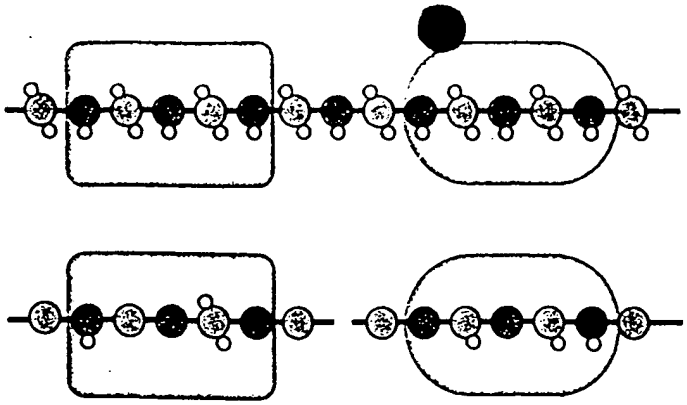


Figure 3. Hidden specificity in protein binding. Heparin is able to bind a large number of proteins via its default-modified disaccharide repeats, as illustrated by the binding of two distinct proteins to apparently similar heparin structures (upper complexes). However, the identification of minimal protein binding domains in HS suggests that only some of the sulfate groups present in the default-modified sequence will actually participate in a given interaction (lower complexes). Contrary to the default-modified sequence, such minimal sequences are protein-selective, such that the HS sequence in the lower left complex is unable to bind the protein on the right. For further information, see the text.

direct binding of heparin to the receptor itself (22); indeed, FGF receptor 4 can be activated by heparin, even in the absence of growth factor (23). The effects of selectively O-desulfated heparin preparations in FGF-2-dependent cell proliferation assays suggested that both IdoA 2-O- and GlcN 6-O-sulfate groups were needed for activity (20), contrary to growth factor binding alone, which required 2-O-sulfate groups only (see above). These and other (24) findings were interpreted in terms of a ternary complex, with FGF-2 and its receptor binding to adjacent, distinct sites on the same polysaccharide chain. This model predicts that whereas a GAG chain that contains the appropriately spaced growth factor binding and receptor binding sequences will promote the FGF-induced cellular response, other species with only one of the sequences, or with incorrectly spaced binding regions, will inhibit the response. Domain spacing, in turn, is determined by the length of any intervening sequence and further influenced by the conformational flexibility of such sequences.

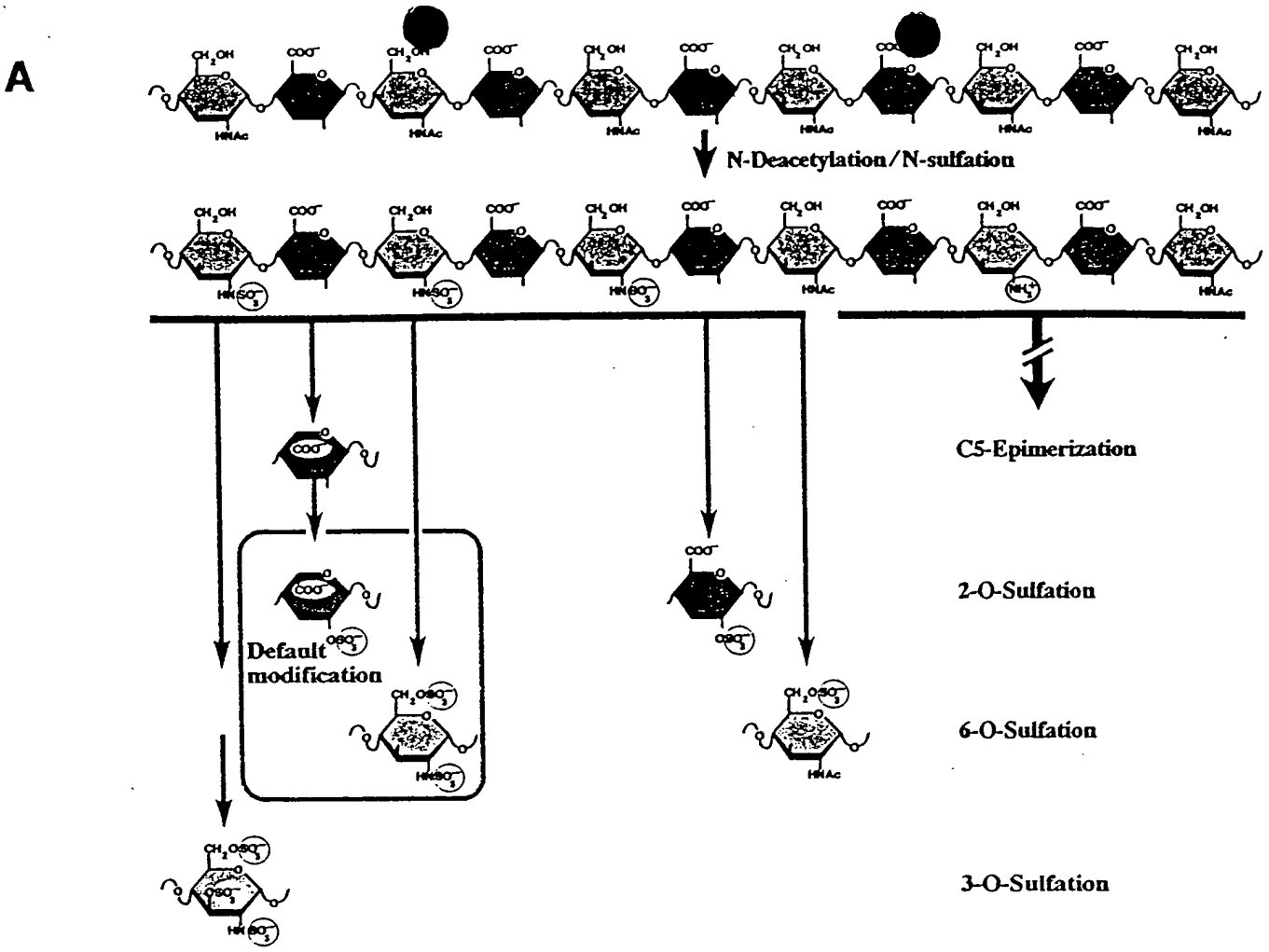
Finally, we consider the possibility of different functional GAG domains residing in different HS chains that are bound to the same core protein (Fig. 2*C*). PGs differ from each other both in the number of potential HS attachment sites and the location of such sites along the protein backbone (2, 3). In glypican, for example, the HS attachment sites appear to be predominantly located close to each other, between the membrane-bound domain and a large extracellular globular domain, whereas syndecans 1 and 3 have more extended core proteins that may carry HS both at the proximal and distal ends of their extracellular domains. The latter arrangement could conceivably facilitate the differential interaction of separate HS chains with several proteins concomitantly. Indeed, syndecan-1, immobilized by interacting through one of its HS chains with fibronectin or collagen, retains its ability to bind FGF-2. By contrast, free HS chains derived from syndecan-1 can bind to only one of the two proteins at a time (25), suggesting a functional versatility for intact PG that is not expressed by the individual HS chain.

POLYSACCHARIDE BIOSYNTHESIS

The biosynthesis of heparin and HS and the regulatory mechanisms required to generate different saccharide sequences of defined structure are only partly understood. Lacking any "code" that specifies such sequences, we need to characterize in detail the enzymes that catalyze the assembly of GAG chains, their concerted mode of action, and their subcellular organization. This discussion will focus on the recent development of the area; for references to older work, see ref 1.

Polysaccharide chain initiation

The enzymes responsible for GAG biosynthesis are located largely in the Golgi apparatus. A tetrasaccharide "linkage region" (-glucuronic acid-galactose-galactose-xylose-) attached to a serine residue in a core protein provides the starting point for polysaccharide chain elongation. The same linkage region is found in PGs carrying glucosaminoglycan (heparin/HS) or galactosaminoglycan (chondroitin sulfate/dermatan sulfate) chains, and there are indications that the same enzymes catalyze the formation of this region in the different types of PGs (26). Once formed, the linkage region will serve as acceptor for the first GlcNAc or GalNAc unit in a reaction that will commit the process toward generation of a glucosamino- or a galactosaminoglycan chain. The hexosaminyl-transferases that add the first GlcNAc/GalNAc units to the linkage region appear to differ from those involved in actual chain elongation, but the factors that determine whether a GlcNAc or a GalNAc unit is to be added remain unclear. Peptide sequence motifs close to GAG-substituted serine residues have been implicated as a signal for the addition of a GlcNAc unit to the linkage tetrasaccharide, thus initiating heparin/HS formation (ref 27 and references therein); an α-GlcNAc transferase catalyzing this reaction has been demonstrated (28). The absence of such a signal would lead to "default" substitution of the linkage region with a GalNAc unit, followed by chondroitin formation. Unexpectedly, transfer of a GalNAc residue to the same tetrasaccharide structure, catalyzed by enzymes present either in bovine fetal serum (29) or in mouse mastocytoma tissue (K. Lidholt, M. Fjelstad, U. Lindahl, T. Ogawa and K. Sugahara, unpublished results), resulted in the incorporation of the α-anomeric sugar rather than the β-GalNAc unit occurring in this position in native chondroitin or dermatan sulfate. Although the significance of this finding is unclear, it is conceivable that the α-GalNAc unit may serve as a general stop signal that prevents further GAG formation. Likewise, the role of the sulfate substituents located at the galactose



residues linked to chondroitin and dermatan sulfate chains, but lacking in those attached to heparin and HS, is not understood (see ref 29 for references).

Formation of the polysaccharide chain

After completion of the tetrasaccharide linkage region, the GAG chain proper is formed by alternating transfer of GlcA and GlcNAc monosaccharide units from the corresponding UDP-sugar nucleotides to the nonreducing termini of nascent chains. This process can be demonstrated in cell-free systems (microsomal preparations) in the absence of subsequent polymer-modification reactions. However, chain elongation is promoted by concomitant N-sulfation, and it is currently believed that, in the intact cell, this and other modification reactions (see below) occur while the chain is still being elongated (30) (**Fig. 4*C*** and **Fig. 5**).

Modification of the $(GlcA\text{-}GlcNAc)_n$ polymer is initiated by N-deacetylation and N-sulfation of GlcNAc units, the latter step as well as the subsequent sulfotransferase reactions requiring 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a sulfate donor. The pathway previously referred to as "default modification" further involves C5-epimerization of GlcA to IdoA units, which are then O-sulfated at C2, and finally O-sulfation of $GlcNSO_3$ units at C6 (Fig. 4). Deviations from this pathway may be due to either restricted or further extended modification. Reactions of the latter category, which generally involve only minor portions of the polysaccharide chains, include O-sulfation at C2 of GlcA (31) and at C3 of GlcN units (Fig. 4; for references, see ref 1).

The most conspicuous restriction of polymer modification is due to incomplete N-deacetylation/N-sulfation. Because the enzymes that catalyze the C5-epimerization and various O-sulfation reactions all require N-sulfate groups for substrate recognition (within a defined distance from the actual target site), sequences composed of consecutive N-acetylated disaccharide units will be devoid of IdoA and O-sulfate residues (1, 6). Such sequences (blue in Fig. 4) are typical for HS but are rare in heparin chains (1). Given the constraints of the modification

C

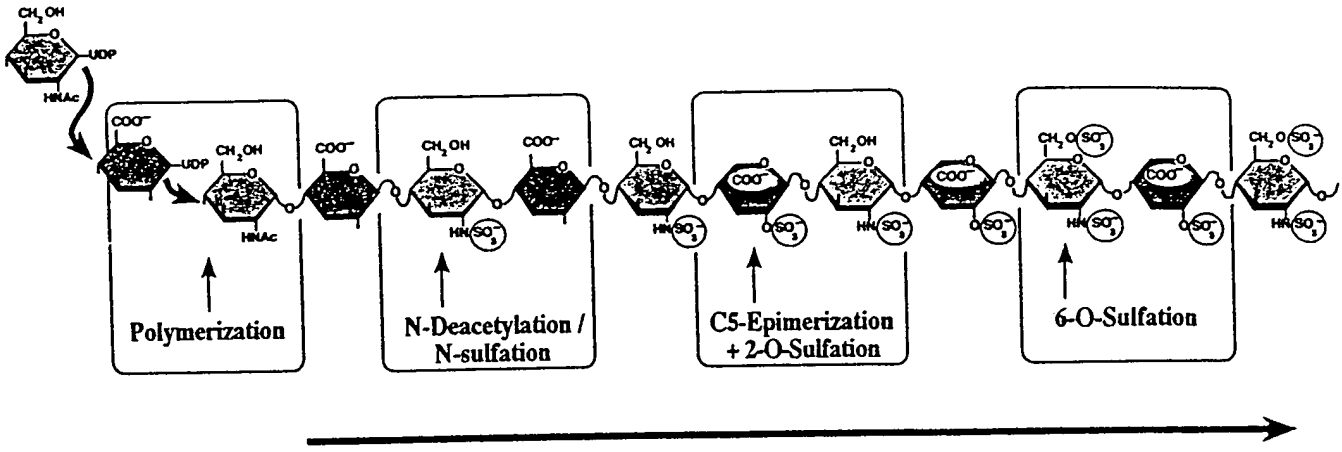


Figure 4. *A)* Polymer-modification reactions involved in the biosynthesis of heparin and HS. The first modification step, N-deacetylation and N-sulfation of GlcN-units, has a key regulatory function because the regions that remain N-acetylated (underlined in blue) will largely escape subsequent modification steps as well (note that an N-acetylated GlcN unit may become 6-O-sulfated, provided that one of the adjacent GlcN residues is N-sulfated). By contrast, N-sulfated regions (underlined in red) are subject to further modification, involving either the default pathway (boxed), a more restricted process (e.g., leaving a nonsulfated IdoA unit), or more extensive modification (e.g., 3-O-sulfation of a $GlcNSO_3$ unit). The position (or positions) of N-unsubstituted GlcN residues (arbitrarily allocated to the N-acetylated region) have not yet been defined. *B)* Scheme showing the domain organization of heparin and HS. The heparin polymer consists of extended, highly modified domains occasionally interspersed by short unmodified domains. HS, on the other hand, typically features unmodified domains of variable length (that may constitute ≥50% of the total polymer) alternating with heterogeneous N-sulfated domains. The four black circles represent the GlcA-Gal-Gal-Xyl linkage region that is bound to a serine residue in the protein core. *C)* Proposed course of polymer formation and default modification in heparin/HS biosynthesis. The indicated coupling between the C5-epimerization and 2-O-sulfation reactions applies to the default modification pathway, but is not mandatory. The direction of passage of the elongating polymer through the modification machinery is indicated by the red arrow, the resultant default-modified chain thus exiting the putative enzyme complex after 6-O-sulfation of $GlcNSO_3$ units. For further information, see the text.

process dictated by substrate specificity and access to a survey of authentic identified structures, a scheme of "permitted" and "forbidden" sequences has been compiled that presumably applies to the entire heparin/HS family (Fig. 2 in ref 1). This scheme does not include the N-unsubstituted GlcN unit, which has yet to be placed in a structural context (see legend to Fig. 4).

Regulation of polymer modification: implications of a model

The scheme in Fig. 4 illustrates the assumed order of the various modification reactions, as deduced essentially from the substrate specificities of the corresponding enzymes. However, the mode of selection of target residues extends beyond a simple matter of substrate specificity. We have no clue as to what mechanism determines whether a particular region of a precursor polysaccharide is going to be N-deacetylated/N-sulfated, and thus subject to further modification, or remain N-acetylated and unmodified. Even within the N-sulfated regions (red in Fig. 4), potential target units often escape modification. For example, a GlcA unit located between two N-sulfated GlcN residues may undergo C5-epimerization to IdoA, but may also remain unchanged. Similarly, 6-O-sulfation of a $GlcNSO_3$ unit located between two IdoA(2-OSO_3) residues is optional. Due to such selectivity, the structural complexity and heterogeneity of the polysaccharide chain will increase through the modification process. The functional relevance of the selection mechanism is apparent, as it provides the basis for the generation of protein binding regions of defined structure.

Our current model depicting the physical course of heparin/HS formation (Fig. 5) features simultaneous elongation and modification of the polysaccharide precursor, in accord with the postulated coupling between the polymerization and N-deacetylation/N-sulfation reactions (30). A glycosyltransferase/N-deacetylase/N-sulfotransferase complex, located at the nonreducing end of the chain, will generate saccharide sequences with N-sulfate or residual N-acetyl groups, which are then subjected to further downstream modification by enzymes that act in a processive fashion along the polysaccharide chain.

Given the main features of this model, we may consider the generation of some structural domains identified in heparin/HS chains. A process in which every disaccharide unit formed would be attacked by each enzyme indicated in Fig. 5 (except the 3-O-sulfotransferase) would lead to a uniform product with the structure expected from default modification (Fig. 1*B*). Deviations from this course involving restricted modification, the hallmark of HS biosynthesis, would require an interrupted, on-off

mode of processive enzyme action. The versatility of this modulation is intriguing. How can we visualize the generation, through the action of a common assembly line, of a polysaccharide chain that contains extended N-acetylated as well as N-sulfated regions of varying length, but also sequences of alternating N-acetylated and N-sulfated disaccharide units that may account for as much as 30% of the total mass of HS chain (6). By what mechanism (or mechanisms) are certain disaccharide units within a contiguous N-sulfated block sequence selected to escape the processive (?) action of one or more of the three major "downstream" enzymes, i.e., the GlcA C5-epimerase, the IdoA 2-O-sulfotransferase, and the GlcN 6-O-sulfotransferase? What causes the apparent separation of

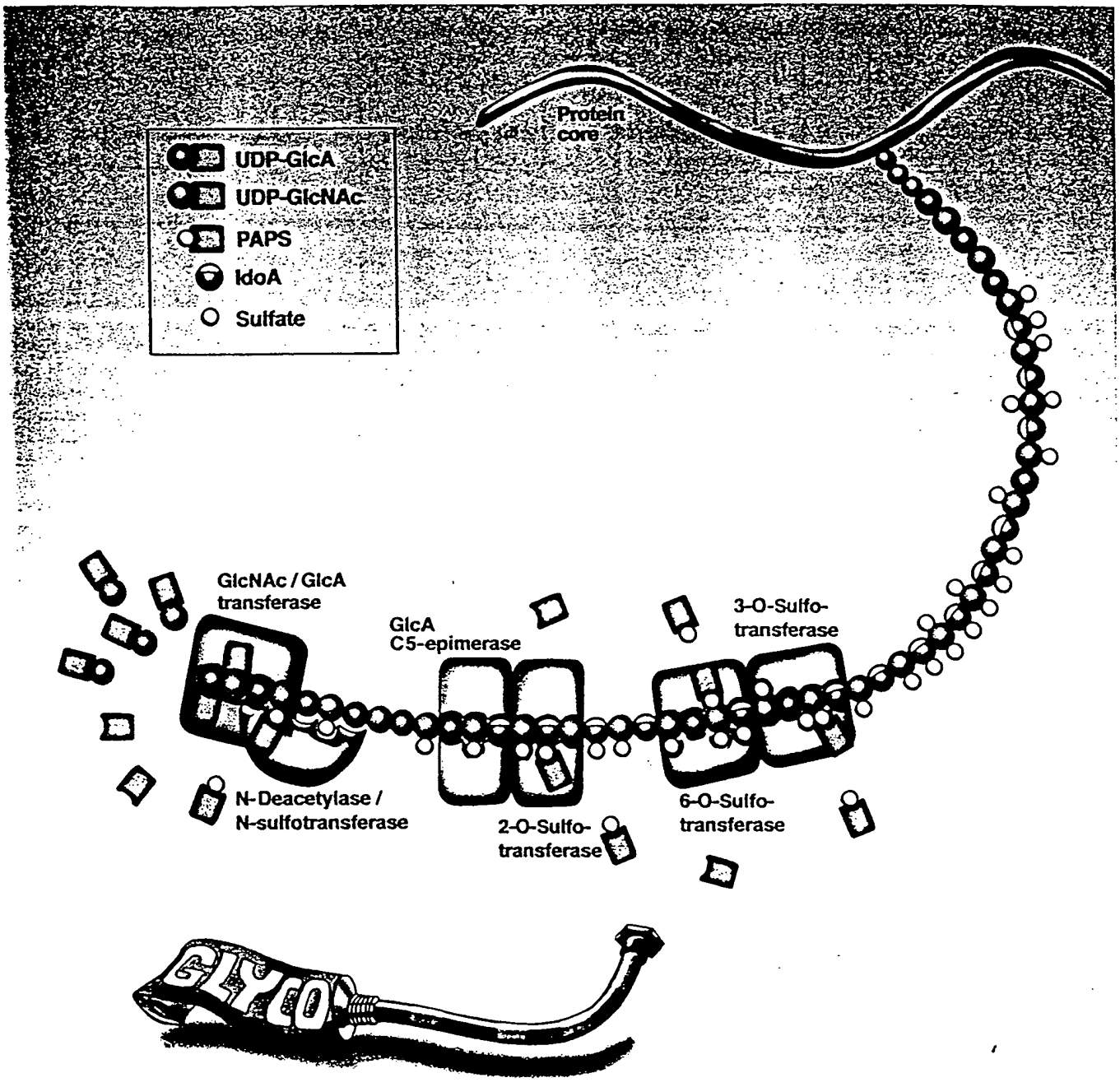


Figure 5. A model of the biosynthetic assembly of heparin/HS in the Golgi complex. A nascent polysaccharide chain, the reducing end bound to a protein core, is shown to traverse the components of the biosynthetic enzyme machinery. The building blocks of the chain, i.e., sugar nucleotides and PAPS, are transported across the Golgi membrane from the cytoplasm where their formation is fueled by cellular ATP. The "polymerase" (GlcNAc/GlcA transferase) adds alternating GlcNAc and GlcA units to the elongating polymer, which is concomitantly, yet sequentially, modified by enzymes catylyzing N-deacetylation and N-sulfation of GlcNAc units, C5-epimerization of GlcA to IdoA units, and O-sulfation at various positions. Enzymes (the "polymerase" and the GlcNAc N-deacetylase/N-sulfotransferase) so far shown to catalyze more than one reaction are indicated. The remaining enzymes have been arbitrarily combined into complexes; indeed, the organization of all enzymes into one major complex appears entirely plausible. The mode of interaction of such a complex with the polysaccharide chain, resulting in the generation of alternating modified (red in Fig. 4) and unmodified (blue in Fig. 4) sequences, is not understood. For further information, see the text. Modified from ref 30.

target sites for 2-O-sulfation, essentially restricted to N-sulfated blocks, and 6-O-sulfation, which occurs within as well as outside these blocks (32; 32a).

The formation of a "unique" marker component is more tangible insofar as it may require the participation of a distinct enzyme. The biosynthesis of the antithrombin binding region in heparin and HS thus is concluded by a GlcN 3-O-sulfotransferase (Figs. 4 and 5) that recognizes as sulfate acceptor a pentasaccharide sequence that differs from the functional binding site (Fig. 1*C*) only by lacking the GlcN 3-O-sulfate target residue (see ref 33). Formation of the acceptor sequence again depends on restricted polymer modification, because the single GlcA unit is essential for substrate recognition. However, analysis of polysaccharide chains lacking the 3-O-sulfate group (hence with low affinity for antithrombin and low blood anticoagulant activity) showed the occurrence of potential 3-O-sulfate acceptor sites that apparently had escaped attack by the enzyme (33). Thus, the GlcN 3-O-sulfotransferase, similar to the IdoA 2-O- and the GlcN 6-O-sulfotransferases, is also subject to regulation based on restricted access to substrate sequences. Overexpression in endothelial cells of a specific HS PG core protein (syndecan-4) that normally carries 3-O-sulfated HS chains led to selective depression of 3-O-sulfation, hence to impeded formation of HS chains with high affinity for antithrombin (34). It was proposed that the increased intracellular levels of syndecan-4 might act by perturbing the functional coordination of the biosynthetic enzymes. Loosening of the interaction between the 3-O-sulfotransferase and its polysaccharide substrate (Fig. 5) would be expected to interfere with 3-O-sulfation, as the enzyme was found to be strongly inhibited by other, more commonly occurring, saccharide sequences, such as the product of default modification (Fig. 1*B*) (33).

2-O-Sulfation of GlcA units has been demonstrated in a cell-free system and appears to occur concomitantly with the sulfation of IdoA, adjacent to at least one N-sulfated GlcN residue (Fig. 4; see ref 1). The mechanism behind the selection of certain GlcA units for 2-O-sulfation is unknown.

Proteins involved in the biosynthetic process

The proteins required to form a heparin or a HS PG include the appropriate core protein, enzymes that catalyze the formation and modification of GAG chains, and any auxiliary proteins that may be involved in the process.

Core proteins

The various core proteins known to carry heparin (serglycin) or HS (syndecans, perlecan, glypicans, and others) GAG chains have been discussed in previous reviews (1, 3, 4, 35, 36).

Enzymes

A deeper understanding of PG biosynthesis, and particularly its regulation, will depend on knowledge regarding the molecular characteristics of the enzymes involved and their genetics. Such information is accumulating due to purification and cloning of the enzymes, as well as through chemical mutagenesis of HS-producing cells (26). None of the enzymes involved in forming the linkage region tetrasaccharde sequence has yet been purified and cloned. A CHO cell mutant unable to produce HS was found to be defective with regard to both the GlcA-transferase and the GlcNAc-transferase reactions (37). Because the defect was presumably due to a single mutation, it was tentatively concluded that the two transferase reactions were catalyzed by a single enzyme protein. This assumption was supported by the identification of a ~70 kDa protein in bovine serum that promoted both reactions (38). The enzyme-deficient CHO cell line accumulated a protein-bound pentasaccharide composed of an α-GlcNAc unit bound to the linkage region tetrasaccharide (39), in accord with the notion that the "polymerase" differs from the GlcNAc-transferase that adds the first GlcNAc unit of the chain (28).

The notion of two distinct reactions being catalyzed by a single enzyme implies a rational means of promoting alternating events along a polymer chain. In fact, a similar arrangement applies to the two first polymer modification reactions, N-deacetylation and N-sulfation of GlcNAc units, which are both associated with the same ~110 kDa enzyme (refs 40–42 and references therein). The regulation of these reactions is essential, because the resultant distribution of N-acetyl and N-sulfate groups will control the followi~g modification reactions, and in fact will determine whether the final product will be classified a heparin or a HS. The N-deacetylase/N-sulfotransferase occurs in two distinct forms with partly different catalytic properties. One of these forms, first isolated from mouse mastocytoma (40), was associated with the biosynthesis of heparin, whereas the other, derived from rat liver (41), was implicated in HS generation. Both enzymes have been cloned (40–42), and comparison of the deduced amino-acid sequences showed that although the putative catalytic domains were closely related, other portions—in particular the N-terminal parts—were different. The two enzymes are encoded by transcripts of markedly different size (40, 42) that are related to separate genes (I. Eriksson, M. Kusche Gullberg, L. Kjellén, personal communication). Differential roles for these enzymes in regulating the N-deacetylation/N-sulfation process during heparin/HS biosynthesis were suggested by the finding that transfection of a HS-producing cell line with cDNA endocing the mast-cell N-deacetylase/N-sulfotransferase induced a drastic change of the N-substituent pattern of the HS produced by the cell toward that typical for heparin (43).

A ~52 kDa GlcA C5-epimerase has been purified to homogeneity from bovine liver (44). This enzyme cata-

lyzes the reversible conversion of GlcA to IdoA units, equilibrium favoring retention of the D-*gluco* configuration. However, whereas information on the kinetics of solubilized enzyme preparations may apply in part also to the intact biosynthetic system, the experimental conditions deviate drastically from those pertaining to GAG formation in the living cell. In this process, chain elongation and modification is completed within a few minutes or less, and individual reactions such as the GlcA C5-epimerization would not be allowed to approach equilibrium (45). In fact, studies of the formation of heparin in a mastocytoma microsomal fraction failed to show any "back-epimerization" (from IdoA to GlcA); the intact biosynthetic system is capable of delivering heparin chains in which ≥80% of the total hexuronic acid is IdoA. This remarkable efficiency is obviously, albeit still mysteriously, due to the mode of concerted interaction of the membrane-bound biosynthetic enzymes with their polymeric substrate.

Information regarding the molecular characteristics of the various O-sulfotransferases is still scanty. A ~60 kDa protein fraction, derived from detergent-solubilized mouse mastocytoma tissue, was found to catalyze both IdoA 2-O- and GlcN 6-O-sulfation, suggesting that these two reactions might also be associated with the same enzyme (46). On the other hand, cultured Chinese hamster ovary cells were found to release a GlcN 6-O-sulfotransferase into the medium while IdoA 2-O-sulfotransferase was retained by the cells (47). Purification of the former enzyme yielded two, 52 and 45 kDa, protein fractions. It cannot be concluded at present whether these discrepancies reflect actual differences between O-sulfotransferases that catalyze the corresponding reactions in heparin and HS biosynthesis or proteolytic processing of a common, membrane-bound O-sulfotransferase.

Auxiliary proteins

Scattered observations point indirectly to the involvement of additional auxiliary proteins in the biosynthetic process. The mouse mastocytomal N-deacetylase/N-sulfotransferase thus requires a polycationic cofactor for activity (40), whereas the corresponding rat liver enzyme does not (48). The endogenous polycation in the mast cell, apparently a polypeptide, may be replaced by synthetic polymers in assays of the purified enzyme. Moreover, analysis of cell mutants deficient in production of HS with high affinity for antithrombin implicated a regulatory component believed to somehow coordinate the action of the biosynthetic enzymes involved in generating the specific antithrombin-binding pentasaccharide sequence (49). Undoubtedly there are other, still undetected, proteins with similar regulatory functions. Such proteins need to be isolated, cloned, and characterized along with the biosynthetic enzymes in order to gain a better understanding of PG biosynthesis and its regulation. We may ultimately visualize the generation of artificial biosynthetic machineries, based on recombinant proteins assembled in appropriate membrane systems, that will enable the efficient formation of saccharide chains with specifically tailored structure. FJ

REFERENCES

1. Lindahl, U., Lidholt, K., Spillmann, D., and Kjellén, L. (1994) More to "heparin" than anticoagulation. *Thromb. Res.* **75**, 1–32
2. Kjellén, L., and Lindahl, U. (1991) Proteoglycans: structures and interactions. *Annu. Rev. Biochem.* **60**, 443–475
3. David, G. (1993) Integral membrane heparan sulfate proteoglycans. *FASEB J.* **7**, 1023–1030
4. Iozzo, R. V. (1994) Perlecan: a gem of a proteoglycan. *Matrix Biol.* **14**, 203–208
5. Spillmann, D., and Lindahl, U. (1994) Glycosaminoglycan-protein interactions: a question of specificity. *Curr. Opin. Struct. Biol.* **4**, 677–682
6. Gallagher, J. T., Turnbull, J. E., and Lyon, M. (1992) Patterns of sulphation in heparan sulphate: polymorphism based on a common structural theme. *Int. J. Biochem.* **24**, 553–560
7. Parthasarathy, N., Goldberg, I. J., Sivaram, P., Mulloy, B., Flory, D. M., and Wagner, W. D. (1994) Oligosaccharide sequences of endothelial cell surface heparan sulfate proteoglycan with affinity for lipoprotein lipase. *J. Biol. Chem.* **269**, 22391–22396
8. Bourin, M.-C., and Lindahl, U. (1993) Glycosaminoglycans and the regulation of blood coagulation. *Biochem. J.* **289**, 313–330
9. Maccarana, M., and Lindahl, U. (1993) Mode of interaction between platelet factor 4 and heparin. *Glycobiology* **3**, 271–277
10. Maccarana, M., Casu, B., and Lindahl, U. (1993) Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. *J. Biol. Chem.* **268**, 23898–23905
11. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science* **271**, 1116–1120
12. Ishihara, M. (1994) Structural requirements in heparin for binding and activation of FGF-1 and FGF-4 are different from that for FGF-2. *Glycobiology* **4**, 817–824
13. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) Structure of heparan sulphate from human brain, with special regard to Alzheimer's disease. *Biochem. J.* **306**, 177–184
14. Fedarko, N. S., and Conrad, H. E. (1986) A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J. Cell Biol.* **102**, 587–599
15. Busch, S. J., Martin, G. A., Barnhart, R. L., Mano, M., and Cardin, A. D. (1992) Trans-repressor activity of nuclear glycosaminoglycans on Fos and Jun/AP-1 oncoprotein-mediated transcription. *J. Cell Biol.* **116**, 31–42
16. van den Born, J., Gunnarsson, K., Bakker, M. A. H., Kjellén, L., Kusche-Gullberg, M., Maccarana, M., Berden, J. H. M., and Lindahl, U. (1995) Presence of N-unsubstituted glucosamine units in native heparan sulfate revealed by a monoclonal antibody. *J. Biol. Chem.* **270**, 31303–31309
17. Norgard-Sumnicht, K., and Varki, A. (1995) Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. *J. Biol. Chem.* **270**, 12012–12024
18. Lortat-Jacób, H., Turnbull, J. E., and Grimaud, J.-A. (1995) Molecular organization of the interferon γ-binding domain in heparan sulphate. *Biochem. J.* **310**, 497–505
19. Groothenhuis, P. D. J., Westerduin, P., Meuleman, D., Petitou, M., and van Boeckel, C. A. A. (1995) Rational design of synthetic heparin analogues with tailor-made coagulation factor inhibitory activity. *Nature Struct. Biol.* **2**, 736–739
20. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) Activating and inhibitory heparin sequences for FGF-2 (basic FGF). Distinct requirements for FGF-1, FGF-2, and FGF-4. *J. Biol. Chem.* **268**, 23906–23914
21. Heldin, C.-H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213–223
22. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science* **259**, 1918–1921
23. Cao, G., and Goldfarb, M. (1995) Heparin can activate a receptor tyrosine kinase. *EMBO J.* **14**, 2183–2190
24. Pantoliano, M. W., Horlick, R. A., Springer, B. A., van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) Multivalent ligand-receptor binding interactions in the fibroblast growth factor system produce a cooperative growth factor and heparin mechanism for receptor dimerization. *Biochemistry* **33**, 10229–10248
25. Salmivirta, M., Heino, J., and Jalkanen, M. (1992) Basic fibroblast growth factor-syndecan complex at cell surface or immobilized to matrix promotes cell growth. *J. Biol. Chem.* **267**, 17606–17610

26. Esko, J. D. (1991) Genetic analysis of proteoglycan structure, function and metabolism. *Curr. Opin. Cell Biol.* **3**, 805–816
27. Zhang, L., David, G., and Esko, J. D. (1995) Repetitive Ser-Gly sequences enhance heparan sulfate assembly in proteoglycans. *J. Biol. Chem.* **270**, 27127–27135
28. Fritz, T. A., Gabb, M. M., Wei, G., and Esko, J. D. (1994) Two N-acetylglucosaminyltransferases catalyze the biosynthesis of heparan sulfate. *J. Biol. Chem.* **269**, 28809–28814
29. Kitagawa, H., Tanaka, Y., Tsuchida, K., Goto, F., Ogawa, T., Lidholt, K., Lindahl, U., and Sugahara, K. (1995) N-Acetylgalactosamine (GalNAc) transfer to the common carbohydrate-protein linkage region of sulfated glycosaminoglycans. Identification of UDP-GalNAc:chondro-oligosaccharide α-N-acetylgalactosaminyltransferase in fetal bovine serum. *J. Biol. Chem.* **270**, 22190–22195
30. Lidholt, K., and Lindahl, U. (1992) Biosynthesis of heparin. The D-glucuronosyl- and N-acetyl-D-glucosaminyltransferase reactions and their relation to polymer modification. *Biochem. J.* **287**, 21–29
31. Kusche, M., and Lindahl, U. (1990) Biosynthesis of heparin. O-Sulfation of D-glucuronic acid units. *J. Biol. Chem.* **265**, 15403–15409
32. Sanderson, P. N., Nieduszynski, I. A., and Huckerby, T. N. (1983) Very-high-field n.m.r. studies of bovine lung heparan sulphate oligosaccharides produced by nitrous acid deaminative cleavage. *Biochem. J.* **211**, 677–682
32a. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, V. (1996) Domain structure of heparan sulfates from bovine organs. *J. Biol. Chem.* **271**, 17804–17810
33. Razi, N., and Lindahl, U. (1995) Biosynthesis of heparin/heparan sulfate. The D-glucosaminyl 3-O-sulfotransferase reaction: target and inhibitor saccharides. *J. Biol. Chem.* **270**, 11267–11275
34. Shworak, N. W., Shirakawa, M., Colliec-Jouault, S., Liu, J., Mulligan, R. C., Birinyi, L. K., and Rosenberg, R. D. (1994) Pathway-specific regulation of the synthesis of anticoagulantly active heparan sulfate. *J. Biol. Chem.* **269**, 24941–24952
35. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M., Spring, J., Gallo, R., and Lose, E. (1992) Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **8**, 333–364
36. Salmivirta, M., and Jalkanen, M. (1995) Syndecan family of cell surface proteoglycans: developmentally regulated receptors for extracellular effector molecules. *Experientia* **51**, 863–872
37. Lidholt, K., Weinke, J. L., Kiser, C. S., Lugemwa, F. N., Bame, K. J., Cheifetz, S., Massagué, J., Lindahl, U., and Esko, J. D. (1992) A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA* **89**, 2267–2271
38. Lind, T., Lindahl, U., and Lidholt, K. (1993) Biosynthesis of heparin/heparan sulfate. Identification of a 70-kDa protein catalyzing both the D-glucuronosyl- and the N-acetyl-D-glucosaminyltransferase reactions. *J. Biol. Chem.* **268**, 20705–20708
39. Zhang, L., and Esko, J. D. (1995) Accumulation of a pentasaccharide terminating in α-N-acetylglucosamine in an animal cell mutant defective in heparan sulfate biosynthesis. *J. Biol. Chem.* **270**, 12557–12562
40. Eriksson, L., Sandbäck, D., Ek, B., Lindahl, U., and Kjellén, L. (1994) cDNA cloning and sequencing of mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase, an enzyme involved in the biosynthesis of heparin. *J. Biol. Chem.* **269**, 10438–10443
41. Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C. B. (1992) Molecular cloning and expression of rat liver N-heparan sulfate sulfotransferase. *J. Biol. Chem.* **267**, 15744–15750
42. Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J., and Ishihara, M. (1994) Molecular cloning and expression of a glycosaminoglycan N-acetylglucosaminyl N-deacetylase/N-sulfotransferase from a heparin-producing cell line. *J. Biol. Chem.* **269**, 2270–2276
43. Cheung, W.-F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U., and Kjellén, L. (1996) Expression of the mouse mastocytoma glucosaminyl N-deacetylase/N-sulfotransferase in human kidney 293 cells results in increased N-sulfation of heparan sulfate. *Biochemistry* **35**, 5250–5256
44. Campbell, P., Hannesson, H. H., Sandbäck, D., Rodén, L., Lindahl, U., and Li, J.-p. (1994) Biosynthesis of heparin/heparan sulfate. Purification of the D-glucuronyl C-5 epimerase from bovine liver. *J. Biol. Chem.* **269**, 26953–26958
45. Lindahl, U. (1989) Biosynthesis of heparin and related polysaccharides. *Heparin: Chemical and Biological Properties, Clinical Applications*, (Lane, D. A., and Lindahl, U., eds) pp. 159–189, Edward Arnold, London
46. Wlad, H., Maccarana, M., Eriksson, I., Kjellén, L., and Lindahl, U. (1994) Biosynthesis of heparin. Different molecular forms of O-sulfotransferases. *J. Biol. Chem.* **269**, 24538–24541
47. Habuchi, H., Habuchi, O., and Kimata, K. (1995) Purification and characterization of heparan sulfate 6-sulfotransferase from the culture medium of chinese hamster ovary cells. *J. Biol. Chem.* **270**, 4172–4179
48. Wei, Z., Swiedler, S. J., Ishihara, M., Orellana, A., and Hirschberg, C. B. (1993) A single protein catalyzes both N-deacetylation and N-sulfation during the biosynthesis of heparan sulfate. *Proc. Natl. Acad. Sci. USA* **90**, 3885–3888
49. Colliec-Jouault, S., Shworak, N. W., Liu, J., de Agostini, A. I., and Rosenberg, R. D. (1994) Characterization of a cell mutant specifically defective in the synthesis of anticoagulantly active heparan sulfate. *J. Biol. Chem.* **269**, 24953–24958